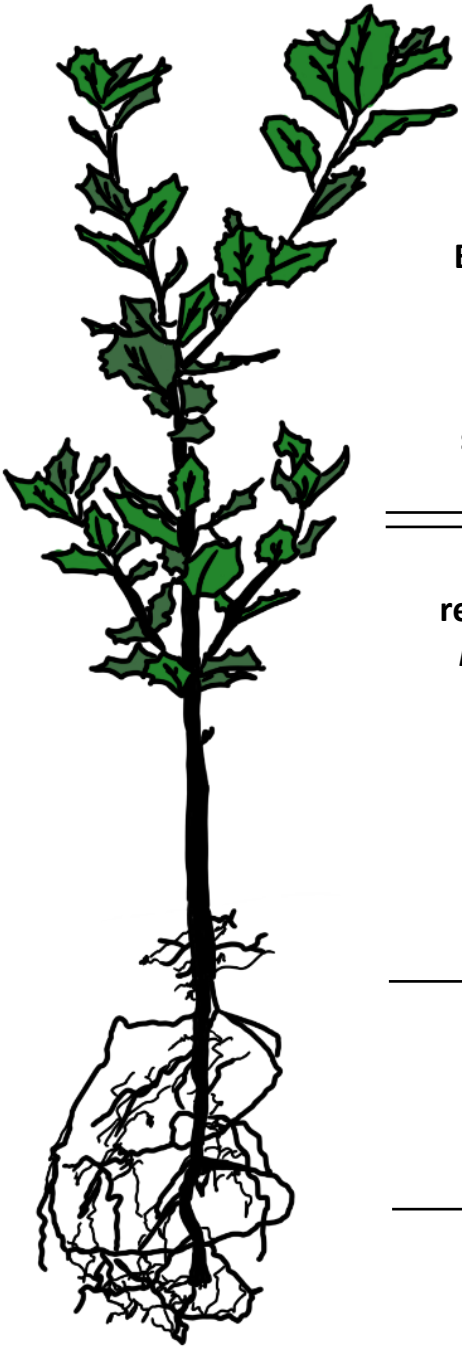

UNIVERSIDAD D CÓRDOBA

Estudios de variabilidad en la respuesta de la encina (*Quercus ilex* L.) a estreses asociados al síndrome de la seca: Sequía y *Phytophthora cinnamomi*

Studies of variability in the response of holm oak (*Quercus ilex* L.) to stresses associated with the decline syndrome: Drought and *Phytophthora cinnamomi*

Tesis doctoral

Bonoso San Eufrasio Martínez

Directoras:

María Dolores Rey Santomé

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Tutor:

Jesús V. Jorrín Novo

Universidad de Córdoba

Junio, 2021

Programa de doctorado en Ingeniería Agraria, Alimentaria, Forestal y del Desarrollo Rural Sostenible

TITULO: *Studies of variability in the response of holm oak (Quercus ilex L.) to stresses associated with the decline syndrome: Drought and Phytophthora cinnamomi*

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Programa de Doctorado en Ingeniería Agraria, Alimentaria, Forestal y del Desarrollo Rural Sostenible por la Universidad de Córdoba y la Universidad de Sevilla

June 2021

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INFORME RAZONADO DE LAS DIRECTORAS DE LA TESIS

La presente Tesis Doctoral, "Estudios de variabilidad en la respuesta de la encina (*Quercus ilex* L.) a estreses asociados al síndrome de la seca: Sequía y *Phytophthora cinnamomi*." ha sido realizada por Bonoso San Eufrasio Martínez, durante los años 2019 a 2021, dentro del "Programa de Doctorado de la Universidad de Córdoba "Ingeniería Agraria, Alimentaria, Forestal y del Desarrollo Rural Sostenible". Ha sido dirigida por las Doctoras María Dolores Rey Santomé y María Ángeles Castillejo Sánchez, de la Universidad

de Córdoba, miembros del grupo de investigación AGR-164; Bioquímica, Proteómica, y Biología de Sistemas Vegetal y Agroforestal. Forma parte del proyecto de investigación: "Seleccion asistida por marcadores moleculares de genotipos elite y tratamiento con activadores de defensa: dos aproximaciones biotecnologicas al problema de la seca en encina. PID2019-109038RB-I00" (Proyectos de I+D+i, en el marco de los Programas Estatales de Generación de Conocimiento y Fortalecimiento Científico y Tecnológico del Sistema de I+D+i y Orientada a los Retos de la Sociedad)

Cumple los requisitos exigidos para su presentación y defensa. El manuscrito se ha escrito en inglés, lo que dará mayor visibilidad al trabajo.

La trayectoria de Bonoso San Eufrasio durante este periodo de formación ha sido buena, habiendo logrado resultados de interés y publicaciones científicas en revistas indexadas (Q1). Su grado de formación se ajusta a los objetivos propuestos en el programa de Doctorado, tanto a nivel científico-técnico como formativo, tal y como queda reflejado en su *curriculum vitae.*

El trabajo de Tesis se ha centrado en una especie de enorme importancia, tanto económica como medioambiental, para Andalucía, la encina. Tuvo como objetivo el estudio de uno de los problemas actuales del bosque mediterráneo y de la dehesa, el síndrome de la seca, que está ocasionando una creciente pérdida de arbolado y degradación de dichos ecosistemas. En concreto se ha centrado en los dos factores de mayor incidencia en el citado síndrome, la sequía y la infección por *Phytophthora cinnamomi.* Propone

actuaciones biotecnológicas en los programas de mejora de la especie, basadas en el análisis de la variabilidad fenotípica, su caracterización molecular y el desarrollo de marcadores para la selección de genotipos élite resilientes. Será de gran utilidad en la planificación de programas de reforestación en un escenario de cambio climático.

El trabajo realizado ha supuesto un avance en los conocimientos del efecto y la respuesta de especies del género *Quercus*, y más concretamente *Q. ilex*, a factores ambientales adversos de tipo biótico y abiótico. El fenotipo resiliente y la respuesta de resiliencia se ha caracterizado en individuos de diferentes procedencias andaluzas a nivel morfológico, fisiológico, bioquímico y proteómico. Todo ello ha supuesto un esfuerzo importante en la optimización de técnicas que pueden ser utilizadas en la identificación temprana de genotipos élites, técnicas que están siendo transferidas al sector productivo.

La Tesis ha dado lugar a las siguientes publicaciones:

San-Eufrasio, B.; Sánchez-Lucas, R.; López-Hidalgo, C.; Guerrero-Sánchez, V.M.; Castillejo, M.Á.; Maldonado-Alconada, A.M.; Jorrín-Novo, J.V.; Rey, M.-D. Responses and Differences in Tolerance to Water Shortage under Climatic Dryness Conditions in Seedlings from *Quercus* spp. and Andalusian *Q. ilex* Populations. *Forests*, 2020, *11*, 707, doi:10.3390/f11060707.

San-Eufrasio, B.; Bigatton, E.D.; Guerrero-Sánchez, V.M.; Chaturvedi, P.; Jorrín-Novo, J.V.; Rey, M.-D.; Castillejo, M.Á. Proteomics Data Analysis for the Identification of Proteins and Derived Proteotypic Peptides of Potential Use as Putative Drought Tolerance Markers for *Quercus ilex*. *International Journal of Molecular Sciences*, 2021, *22*, 3191, doi:10.3390/ijms22063191.

San-Eufrasio, B.; Castillejo, M.A.; Labella-Ortega, M.; Ruiz-Gómez, F. J.; Navarro-Cerrillo, R. M.; Jorrín-Novo, J. V.; Rey, M. D. Effect and response to combined *Phytophthora cinnamomi* and drought in *Quercus ilex* subsp. *ballota* [Desf.] Samp. seedlings from three contrasting Andalusian populations. *Frontiers in Plant Science*. Under evaluation.

El trabajo ha sido también publicado en capítulos de libro y ha sido presentado en diferentes congresos y reuniones científicas:

Gomez-Galvez, I.; Sanchez-Lucas, R.; **San-Eufrasio, B.**; Rodríguez de Francisco, L. E.; Maldonado-Alconada, A. M.; Fuentes-Almagro, C.; Castillejo, M. A. Optimizing Shotgun Proteomics Analysis for a Confident Protein Identification and Quantitation in Orphan Plant Species: The Case of Holm Oak (*Quercus ilex*). 2020, 2139, 157-168. *Methods in Molecular Biology. Plant Proteomics*. Springer Nature, New York, U.S.A.

San-Eufrasio, B.; Jorrín-Novo, J. V.; Castillejo, M. A.; Rey, M. D. Programa de mejora en encina: Selección de genotipos élite tolerantes a la Seca y resilientes al cambio climático. Seminarios de actualidad sobre los

retos de la investigación en la ingeniería agraria, alimentaria, forestal y del desarrollo rural sostenible. VIII Congreso Científico de Investigadores en Formación de la Universidad de Córdoba, 2020.

San-Eufrasio, B.; Bigatton, E. D.; Guerrero-Sánchez, V. M.; Jorrín-Novo, J. V.; Castillejo, M. A. A targeted proteomics approach for the selection of peptides to be used as markers of tolerance to drought in *Quercus ilex*. VIII Congreso SEProt Oviedo, 2020.

Escandón, M.; Rey, M. D.; Labella-Ortega, M.; Tienda-Parrilla, M.; **San-Eufrasio, B.**; Guerrero-Sánchez, V. M.; Maldonado-Alconada, A. M.; Castillejo, M. A.; Jorrín-Novo, J. V. Unravelling *Quercus ilex* biology throught -omics approaches and its translation to the search of elite, resilient, genotypes. XV reunión de biología molecular de plantas, 2020.

Castillejo, M. A.; **San-Eufrasio, B.**; Jorrín-Novo, J. V.; Rey, M. D. A shotgun proteomics approach for the study of the effect and responses to combined drought and *Phytophthora cinnamomi* in *Quercus ilex* subsp. *ballota* [Desf.] Samp. seedlings from two contrasting Andalusian populations, 4th INPPO conference, 2021.

Castillejo, M. A.; **San-Eufrasio, B.**; Bigatton, E. D.; Guerrero-Sánchez, V. M.; Rey, M. D.; Jorrín-Novo, J. V. Targeted post-acquisition proteomics as an approach for the search of proteins and peptides to be used as markers of tolerance to drought in *Quercus ilex*, 4th INPPO conference, 2021.

El estudio y los resultados son totalmente novedosos para la encina. El trabajo ha contribuido a la formación de Bonoso San Eufrasio en el campo de las especies forestales y en lás áreas de Fisiología, Bioquímica y Biología Molecular y de la respuesta de las plantas a estreses bióticos y abióticos.

El número de actividades formativas realizadas por Bonoso San Eufrasio se ha ajustado a las exigidas por el Programa de Doctorado, las sugeridas por sus directores, y las seguías por propia iniciativa. En concreto ha realizado las siguientes:

- Actividades Formativas Obligatorias:

Seminarios de actualidad sobre los retos de la investigación en la ingeniería agraria, alimentaria, forestal y del desarrollo rural sostenible (VIII Congreso Científico de Investigadores en Formación de la Universidad de Córdoba). Charla titulada: Programa de mejora en encina: Selección de genotipos élite tolerantes a la Seca y resilientes al cambio climático.

Visitas a centros, grandes instalaciones y laboratorios de investigación del sector privado y del público (Estación experimental el Zaidín). Explicación sobre un ejemplo de centro de investigación. Actividades realizadas y visita por los grupos de investigación que lo forma.

Mejora de la empleabilidad y orientación laboral. El doctorando recibió varios seminarios relacionados con la búsqueda de empleo y posibles salidas profesionales cuando acaba el doctorado.

- Otras actividades formativas:

Jornada Formativa Doctoral sobre el doctorado en la Universidad de Córdoba, marco normativo, procesos y procedimientos 2019. Explicación sobre trámites, plazos y documentación relacionada tanto con el programa de doctorado al que pertenezco, como ejemplos de otros programas.

Jornada técnica sobre *Phytophthora* spp. (podredumbre radical) en las dehesas. Situación actual del síndrome de la Seca, y posibles soluciones actuales y en un futuro próximo.

Apoyo en la impartición de prácticas de laboratorio en el grado de ingeniería agronómica.

- Actividades divulgativas:

Participación en actividad de divulgación científica mediante la explicación de un ejemplo de proyecto biotecnológico en el proyecto de innovación docente presentado por el grupo de investigación Bioquímica, proteómica y biología de sistemas vegetal y agroforestal en el año 2019.

Paseo por la ciencia 2019. Jornadas de divulgación.

La noche europea de los investigadores 2019. Jornadas de divulgación.

La noche europea de los investigadores 2020. Jornadas de divulgación.

Bonoso San Eufrasio ha mostrado ganas de aprender e interés y por la actividad investigadora. Ha estado dispuesto, a compartir sus conocimientos y experiencias con otros estudiantes de grado, máster y doctorado, ayudándoles generosamente en la realización de las correspondientes Tesis.

Finalizaremos este informe señalando que el trabajo de Bonoso San Eufrasio durante su periodo de doctorado ha transcendido el ámbito de la presente tesis, participando en actividades docentes.

Por todo ello, se autoriza la presentación de la tesis doctoral:

Córdoba, 7 de junio de 2021

Firma de las directoras:

REY SANTOME MARIA DOLORES - 76084037Z

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Fdo. María Dolores Rey Santomé

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Fdo. María Ángeles Castillejo Sánchez

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DOCTORANDO/A: Bonoso San Eufrasio Martínez

INFORME RAZONADO DEL TUTOR

La presente Tesis Doctoral, "Estudios de variabilidad en la respuesta de la encina (*Quercus ilex* L.) a estreses asociados al síndrome de la seca: Sequía y *Phytophthora cinnamomi*." ha sido realizada por Bonoso San Eufrasio Martínez, durante los años 2019 a 2021, dentro del "Programa de Doctorado de la Universidad de Córdoba "Ingeniería Agraria, Forestal, Alimentaria y Desarrollo Rural Sostenible ". Ha sido dirigida por las Doctoras María Dolores Rey Santomé y María Ángeles Castillejo Sánchez, de la Universidad

de Córdoba, miembros del grupo de investigación AGR-164; Bioquímica, Proteómica, y Biología de Sistemas Vegetal y Agroforestal. Forma parte del proyecto de investigación: “Seleccion asistida por marcadores moleculares de genotipos elite y tratamiento con activadores de defensa: dos aproximaciones biotecnologicas al problema de la seca en encina. PID2019-109038RB-I00” (Proyectos de I+D+i, en el marco de los Programas Estatales de Generación de Conocimiento y Fortalecimiento Científico y Tecnológico del Sistema de I+D+i y Orientada a los Retos de la Sociedad).

El Doctorando ha cubierto los objetivos científicos y formativos propuestos en su programa de Tesis. Su trabajo ha permitido profundizar en los conocimientos de la respuesta de la encina a estreses bióticos y abióticos. Los resultados son de gran interés en programas de mejora de la especie basados en la identificación asistida por marcadores moleculares de genotipos élites resilientes al síndrome de la seca. La calidad de los mismos está avalada por dos publicaciones en revistas de impacto. Su grado de formación científica es bueno y es merecedor del Grado de Doctor.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 7 de junio de 2021

Fdo. Jesús Valentín Jorrín Novo

JORRIN NOVO JESUS VALENTIN - 30446605X

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Agradecimientos

Me gustaría expresar mi más sincero agradecimiento a todas las personas y entidades que han participado de alguna manera en la presente tesis doctoral.

A las entidades que la han financiado ya que sin ellas no habría sido posible. Al proyecto "Selección asistida por marcadores moleculares de genotipos élite y tratamiento con activadores de defensa: dos aproximaciones biotecnológicas al problema de la seca en encina. PID2019-109038RB-I00", a la dotación del proyecto RYC-2017-23706 y a la Universidad de Córdoba.

A Jesús, mi tutor y jefe, por su aceptación en el grupo, dedicación e infinita paciencia. A mis directoras Lola y M. Ángeles, por sus conocimientos y el tiempo que me han dedicado. Mari Carmen, por siempre ser tan amable, Anita y Manolo.

Al grupo del profesor Rafael Navarro, Fran y Roberto, también por sus consejos y ayuda.

A mis compañeros de laboratorio, empezando por Fabiola, quien me ayudó en esos primeros momentos y de igual forma, a la Dra. Escandón por su simpatía. A todas aquellas personas que, en mayor o menor medida, me han apoyado estos años: Little Mónica y Diego, Mehtap, Ezequiel y José María, Rosa, Cristina y Victor, Irene, Tamara, M. del Mar, Rubén, Marta y Mercedes, Alejandro, Conchi y Silvia, Haminton, Alvaro, Malika, Daniela, Felix, Carmen y JuanMa.

A Carolina por todo el apoyo que me ha dado y a mis amigos.

A mi familia, mis abuelos, padres y hermana.

Index

Abstract

Resilience[1] to adverse environmental conditions (biotic and abiotic stresses) is one of the main objectives in forest tree breeding programs, and it should be a priority for Holm oak (*Quercus ilex*), considered the increasing mortality of individuals associated to the decline syndrome and the predicted in a climate change scenario. As a non-domesticated species and considering its biological characteristics (long-lived, allogamous, wind-pollinated), the only plausible strategy in Holm oak breeding programs is the selection of resistant and tolerant elite genotypes for clonal propagation and ulterior use in reforestation. Elite or plus genotype trees can be characterized and identified at the morphological, physiological, and molecular (nucleic acids, proteins, and metabolites) levels. The research and characterization of the molecular mechanisms and genes implicated in the response and resilience to stresses will favour the selection of elite genotypes at early stages of the biological cycle, thus speeding breeding programs. In this direction, the present Thesis was planned, which aimed at studying the effect and response

[1] In the present manuscript, the terms resilience, tolerance, and resistance have been used when describing the plant stress-response phenotype. All of them indicate the capacity of the plant to cope, manage, growth and survive under adverse environmental factors, either biotic and abiotic. The opposite general term utilized is susceptibility referring to plants showing severe damage symptoms resulting in death along the experiment. The three terms have been employed without mechanicist implications, physiological or molecular. It is possible that all of them are used here and in the current literature in an ambiguous and confused way. To avoid confusions while reading this Thesis, tolerance refers to the response to abiotic stresses, resistance to biotic, and resilience to a combination of both. A similar use of the three terms can be found in a number of publications, as for example: Thiry *et al.*, 2016.

to individual and/or combined, drought and *Phytophthora cinnamomi* (root rot), stresses in Holm oak. Both stresses constitute the main factors of the decline syndrome and supposed to be one of the main causes of tree mortality in a climate change scenario.

The experiments were performed with seedlings from populations representatives of the different Andalusian provenances. They were subjected to drought stress under high temperature and irradiation (chapter 2 and 3) or to a combination of drought and *P. cinnamomi* inoculation (chapter 4).

The effect and the response to stresses was evaluated at different levels:

i) Morphological: growth, damage symptoms, and seedling dead (Chapters 2 and 4).

ii) Physiological: water content, and photosynthesis (Chapters 2 and 4).

iii) Biochemical: pigments, sugars, amino acids, phenolics, and flavonoids content (Chapters 2 and 4).

iv) Omics: proteomics (Chapters 3 and 4).

From the data on damage symptoms and plant mortality, phenotypic differences (tolerance, resistance, susceptibility) between species, populations and individuals were observed. According to physiological, biochemical, and proteomics data, mechanisms of tolerance and resistance are proposed. From the proteomics data gene products related to the resilient

character are identified, with some of the proteins and derived peptides proposed as molecular markers of resilience.

Quercus ilex intra- and interpopulation phenotypic variability in the response to the studied stresses has been found with differences based on the percentage of damaged and dead seedlings. This percentage should be used as an indicator of the response to drought and *P. cinnamomi*. Under drought conditions, the Eastern populations (Jaen and Granada) showed a lower damage and mortality than the Western ones (Cadiz, Cordoba, and Seville), being characterized as the most tolerant. The combined effects of the *P. cinnamomi* attack and drought stress were more damaging than the effects of pathogen attack or drought stress alone for three populations tested (Seville, Granada, and Almeria), being more severe in Almeria, Eastern population, an area in which the presence of the pathogen has not been constated yet. Granada population showed a better response to the individual and combined stresses, being the population with higher number of surviving seedlings, and supposed to be the most resilient to the decline syndrome.

Different leaf physiological parameters related to water regime and photosynthesis activity have been measured, including relative leaf water content (RLWC), leaf fluorescence and derived PS II quantum yield at dark-adapted state (Qy), net photosynthesis and stomatal conductance. These parameters have been widely utilized in the study of seedling responses to stresses and taken as an estimation of tolerance and resistance in *Q. ilex* and other *Quercus* species. Although RLWC decreased in response to drought to

different degrees depending on the individual and population (the Eastern populations showed a higher RLWC that the Western ones), *Q. ilex* seedlings kept leaf tissue well hydrated, with values above 40% in tolerant individuals. Similar results were obtained in the combined drought and *P. cinnamomi* treatment, although the reduction of RLWC was more accused in the combined stress. Tolerant individuals kept RLWC and turgor above a threshold value of 40%, being one of the key responses to water deprivation stresses.

Under stress conditions, a reduction in photosynthetic activity was observed as indicated by the decrease in the Qy parameter. The decrease was dependent on the stress conditions, duration, and population, being maximum in the combined drought and *P. cinnamomi* treatments, and for the Almeria population. Stomatal closure is a strategy used to diminish water transpiration, prevent leaf water potential, and avoid xylem cavitation under unfavourable conditions. Therefore, stomatal conductance and net photosynthesis were diminished under drought and/or *P. cinnamomi* treatments. The effect observed in *Q. ilex* was also stress-, duration- and population-dependent. The difference in drought response between Western and Eastern populations deduced from the previous parameters discussed above was not observed while using the stomatal conductance and net photosynthesis parameters. Cadiz population, where most dead seedlings were observed under drought conditions, correlated with the lowest RLWC and the highest stomatal conductance, thus suggesting it to be the most

susceptible population among the ones studied.

The content of chlorophylls, carotenoids, amino acids, starch, sugars, phenolics and flavonoids was quantified in control and stressed, non-damaged, seedlings at the end of the experiment (25 and 32 days for the drought and combined stress experiments respectively). In general, photosynthetic pigments remained unchanged under stressing conditions, being in the range of those corresponding to control seedlings, indicating that the photosynthetic molecular machinery was not affected. An increase in the content of sugar, amino acids and total phenolics was observed under drought conditions, which is expected in those tolerant species prone to drought. In the *P. cinnamomi* or combined stress experiment, neither amino acid nor phenolic content was modified, indicating that the response was stress dependent. On the contrary, sugar, flavonoids and starch were increased in inoculated and combined seedlings. Metabolic homeostasis and reorganization of the pathways, from autotrophic to heterotrophic routes, and the increase of stress related (antioxidants, osmotic active compounds) is proposed as a general response to the treatments, with particularities for biotic and abiotic stresses, and with no qualitative, but quantitative ones, differences among provenances and individuals.

The last level of study was the proteomics analysis of the changes in the leaf protein profile in response to individual or combined stresses. It had a double objective, the first one related to the identification of mechanisms and gene products related to the stress-resilient character, and the second one focussed

on the proposal of protein markers to be used in the identification of elite genotypes. For that, a triple strategy was used: gel-based, gel-free or shotgun, and targeted post-acquisition data analysis. Changes in the protein profiles were stress- and individual-dependent, with general tendencies for the different and populations. Variable proteins belonged to photosynthesis, starch, sugar and phenolic metabolism, protein fate, stress-related, and transport. When analysing the functional groups of variable proteins, a general tendency was found, in which an accumulation of stress- and defense-related proteins, antioxidant and phenolic pathway enzymes, and a reduction of those of photosynthesis was observed.

While photosynthetic enzymes were almost not affected under drought conditions, they were less abundant in the combined stress. Proteomics data on starch metabolic enzymes under drought conditions were apparently contradictory, as there were up- and down-accumulated proteins. It may be related to the presence of different types of starch, whether permanent or transitory and that of isoforms involved in their synthesis and mobilization. Key enzymes of the shikimate–phenolic biosynthetic pathways (chalcone synthase and 3-phosphoshikimate 1-carboxyvinyltransferase) were more abundant under drought conditions. Within the protein fate group, synthesis (ribosomal and transcription) was the most represented, showing qualitative and quantitative changes, followed by folding and degradation categories. In general, under adverse conditions, an increase in stress-related proteins was observed, including redox, antioxidant enzymes, and drought and pathogen

responsive.

A panel of 30 proteins and 46 derived peptides, increased in response to drought in at least two out of the four populations surveyed are proposed as markers for this conditions, they belonging to redox, stress-related, synthesis, -folding and degradation, and primary and secondary metabolism functional groups. Two of them, subtilisin and chaperone GrpE were found at increased levels in three populations, which make them especially interesting for validation in subsequent experiments. Out of the total variable proteins found in the combined stress experiment, four are proposed as putative markers of resilience, including an aldehyde dehydrogenase, glucose-6-phosphate isomerase, 50S ribosomal protein L5, and alpha-1,4-glucan-protein synthase [UDP-forming].

Despite the advances in the knowledge of the molecular mechanisms behind the resilience to drought and *P. cinnamomi* generated in the present Thesis, and considering the high variability found in *Q. ilex*, it is important to remind that the proposed mechanisms and markers must be functionally validated by reverse genetics strategies and confirmed for a higher number of populations and individuals before being considered as general for *Q. ilex*. The next step will be to understand the genetic and epigenetic bases of the resilience and the differences between genotypes. In this direction, the recent sequencing of the *Q. ilex* genome carried out in the group will be a key milestone.

Resumen

La resiliencia[2] a condiciones ambientales adversas (estreses bióticos y abióticos) es uno de los principales objetivos de los programas de Mejora Forestal, siendo una prioridad para la encina (*Quercus ilex*), teniendo en cuenta la creciente mortalidad de individuos asociados al Síndrome de la Seca y las previsiones de Cambio Climático. Como especie no domesticada y considerando sus características biológicas (longeva, alógama y polinizada por el viento), la única estrategia plausible en un programa de mejora de la encina es la selección de genotipos élite resistentes y tolerantes a estreses para su posterior propagación clonal y su uso en programas de reforestación. Los genotipos élite o plus pueden caracterizarse e identificarse a nivel morfológico, fisiológico y molecular (ácidos nucleicos, proteínas y metabolitos). La investigación y caracterización de los mecanismos moleculares y genes implicados en la respuesta y resiliencia al estrés favorecerá la selección temprana de genotipos élite, acelerando así los programas de mejora. En este contexto se propuso la presente Tesis, la cual

[2] En el presente manuscrito se han utilizado los términos resiliencia, tolerancia y resistencia para describir el fenotipo de respuesta al estrés en las plantas. Todos ellos indican la capacidad de la planta para hacer frente, gestionar, crecer y sobrevivir bajo factores ambientales adversos, tanto bióticos como abióticos. El término general opuesto que se utiliza es el de susceptibilidad, que se refiere a las plantas que muestran síntomas de daños graves que provocan su muerte a lo largo del experimento. Los tres términos se han empleado sin implicaciones mecanicistas, fisiológicas o moleculares. Es posible que todos ellos se utilicen aquí y en la literatura actual de forma ambigua y confusa. Para evitar confusiones durante la lectura de esta Tesis, la tolerancia se refiere a la respuesta al estrés abiótico, la resistencia al biótico y la resiliencia a una combinación de ambos. Se puede encontrar un uso similar de los tres términos en varias publicaciones, como, por ejemplo: Thiry *et al.*, 2016.

tuvo como principal objetivo estudiar el efecto y respuesta a estreses individuales y/o combinados de sequía y *Phytophthora cinnamomi* (podredumbre radical) en encinas. Ambos estreses constituyen los principales factores del Síndrome de la Seca y una de las posibles causas de mortalidad de los árboles en un escenario de cambio climático.

Los experimentos se realizaron con plántulas de poblaciones de diferentes procedencias andaluzas y fueron sometidos a estrés por sequía en condiciones de temperatura e irradianza elevadas (Capítulo 2) o a una combinación de sequía e inoculación con *P. cinnamomi* (Capítulo 4).
El efecto y la respuesta al estrés se evaluó a diferentes niveles:
i) Morfológico: crecimiento, síntomas de daño y mortandad (capítulos 2 y 4).
ii) Fisiológico: contenido de agua y fotosíntesis (capítulos 2 y 4).
iii) Bioquímico: contenido en pigmentos, azúcares, aminoácidos, fenólicos y flavonoides (capítulos 2 y 4).
iv) Ómico: proteómica (capítulos 3 y 4).

A partir de los datos de daños y mortalidad, se observaron diferencias fenotípicas (tolerancia, resistencia, susceptibilidad) entre especies, poblaciones e individuos. A partir de los análisis fisiológicos, bioquímicos y proteómicos, se proponen mecanismos de tolerancia y resistencia. La proteómica permitió la identificación de genes relacionados con el carácter resiliente, y la identificación de proteínas y péptidos derivados que pueden

ser utilizados como marcadores moleculares de resiliencia.

En base al número de plántulas dañadas y muertas, se encontró una variabilidad fenotípica intra e interpoblacional en la respuesta de *Q. ilex* a los estreses impuestos. El porcentaje de individuos dañados y muertos debe utilizarse como indicador de la respuesta a la sequía y *P. cinnamomi*. En condiciones de sequía, las poblaciones orientales (Jaén y Granada) presentaron menor daño y mortalidad que las occidentales (Cádiz, Córdoba y Sevilla), caracterizándose como las más tolerantes. Los efectos del estrés combinado, *P. cinnamomi* y sequía fueron más intensos que el de los estreses individuales, *P. cinnamomi* o sequía, en las tres poblaciones analizadas (Sevilla, Granada y Almería), siendo la de Almería la más afectada, localizada ésta en una zona donde aún no se ha constatado la presencia del patógeno. La población de Granada mostró una mejor respuesta a los estreses individuales y combinados, siendo la población con mayor número de plántulas vivas, y, por tanto, la más resistente al Síndrome de la Seca.

Se han medido diferentes parámetros fisiológicos de las hojas relacionados con el contenido hídrico y la actividad fotosintética, incluyendo el contenido relativo de agua en hoja (RLWC), la fluorescencia y el rendimiento cuántico de PSII en condiciones de oscuridad (Qy), la fotosíntesis neta y la conductancia estomática. Estos parámetros se han utilizado ampliamente en el estudio de las respuestas de las plántulas al estrés y se han tomado como una estimación de la tolerancia y resistencia en *Q. ilex* y otras especies de

Quercus. Aunque el RLWC disminuyó en respuesta a la sequía en diferentes grados según el individuo y la población (las poblaciones del Este mostraron un RLWC más alto que las del Oeste), las plántulas de *Q. ilex* mantuvieron el tejido foliar bien hidratado, con valores superiores al 40% en individuos tolerantes. Se obtuvieron resultados similares en el tratamiento combinado de sequía y *P. cinnamomi*, aunque la reducción de RLWC fue más acusado. Los individuos tolerantes mantuvieron el RLWC y la turgencia por encima de un valor umbral del 40%, siendo una de las respuestas clave al estrés hídrico.

En condiciones de estrés, se observó una caída en la actividad fotosintética como lo indica la disminución en el parámetro Qy. Dicha caída dependió de las condiciones de estrés, duración y población, siendo máxima en los tratamientos combinados de sequía y *P. cinnamomi*, y para la población de Almería. El cierre estomático es una estrategia utilizada en condiciones desfavorables para disminuir la pérdida de agua, prevenir la caída en el potencial hídrico y evitar la cavitación del xilema. La conductancia estomática y la fotosíntesis neta disminuyeron con los tratamientos de sequía y/o *P. cinnamomi*, siendo el efecto, al igual que el de otros parámetros fisiológicos, dependiente del estrés, la duración y la población. La diferencia observada entre las poblaciones occidentales y orientales en respuesta a la sequía deducida de los parámetros anteriores no se observó al estudiar los parámetros de conductancia estomática y fotosíntesis neta. La población de Cádiz presentó el mayor número de plántulas muertas en condiciones de

sequía, que se correlacionó con el RLWC más bajo y la conductancia estomática más alta, lo que sugiere que es la población más susceptible entre las estudiadas.

El contenido en clorofilas, carotenoides, aminoácidos, almidón, azúcares, fenólicos y flavonoides se cuantificó en el tejido foliar de las plántulas control y estresadas, no dañadas, al final del experimento (días 25 y 32 para los experimentos de sequía y estrés combinado respectivamente). En general, los pigmentos fotosintéticos se mantuvieron sin cambios en condiciones de estrés, estando en el rango de los correspondientes a las plántulas control, lo que indica que la maquinaria molecular fotosintética no se vio afectada. Se observó un aumento en el contenido de azúcares, aminoácidos y compuestos fenólicos en condiciones de sequía, tal y como se ha descrito en otras especies tolerantes a sequía. En el experimento de estrés combinado, no se modificó el contenido de aminoácidos ni de compuestos fenólicos, lo que indica que la respuesta molecular dependió del tipo de estrés. Por el contrario, el contenido en azúcares, almidón y flavonoides aumentó en las plántulas inoculadas y sujetas a estrés combinado. Se propone que durante situaciones de estrés la planta activa mecanismos reguladores de la homeostasis metabólica, mediante la reorganización de rutas con disminución y aumento de, respectivamente, rutas autótrofas a heterótrofas, y el aumento de compuestos de respuesta a estreses (antioxidantes, compuestos activos osmóticos).

El último nivel de estudio llevado a cabo fue el del análisis proteómico para detectar cambios en el perfil proteico de la hoja en respuesta a estreses individuales o combinados. Tuvo un doble objetivo, el primero relacionado con la identificación de mecanismos y productos génicos relacionados con el carácter resiliente/resistente/tolerante al estrés, y el segundo, la identificación y propuesta de marcadores proteicos para ser utilizados en la identificación de genotipos élite. Para eso, se utilizó una triple estrategia proteómica: basada en gel, libre de gel o shotgun, y dirigida, utilizando péptidos proteotípicos. Los cambios en los perfiles proteicos dependieron del tipo de estrés y del individuo, con tendencias generales para las diferentes poblaciones. Las proteínas variables pertenecieron principalmente a los grupos funcionales relacionados con la fotosíntesis, metabolismo de almidón, azúcares y de compuestos fenólicos, síntesis y degradación de proteínas, transporte y estrés. En situaciones de estrés, se observó como tendencia general un aumento en proteínas relacionadas con el estrés y defensa, enzimas antioxidantes y de la ruta de síntesis de fenólicos.

En condiciones de sequía no se vieron cambios apreciables en proteínas y enzimas fotosintéticas; sin embargo, algunas de ellas disminuyeron en condiciones de estrés combinado. Los datos proteómicos sobre las enzimas del metabolismo del almidón en condiciones de sequía fueron aparentemente contradictorios, ya que había proteínas más y menos abundantes. Esta observación puede estar relacionada con la existencia de diferentes tipos de almidón, permanente o transitorio, y de isoformas involucradas en su síntesis

y movilización. Las enzimas clave de la ruta del shikimato-fenólicos (chalcona sintasa y 3-fosfosquimato 1-carboxiviniltransferasa) fueron más abundantes en condiciones de sequía. En general, en condiciones adversas, se observó un aumento de proteínas relacionadas con el estrés, incluidas enzimas redox, antioxidantes y de respuesta a sequía y patógenos.

Se propone como marcadores de tolerancia a la sequía un panel de 30 proteínas y 46 péptidos derivados, que se vieron incrementadas en respuesta a esta condición en al menos dos de las cuatro poblaciones analizadas. Correspondieron a enzimas antioxidantes, relacionadas con el estrés, síntesis, plegamiento y degradación de proteínas, y a enzimas del metabolismo primario y secundario. Dos de ellas, la subtilisina y la chaperona GrpE aumentaron en tres poblaciones, lo que las hace especialmente interesantes para su validación en experimentos posteriores. De las proteínas variables totales encontradas en el experimento de doble estrés, cuatro se proponen como posibles marcadores de resiliencia, incluida una aldehído deshidrogenasa, una glucosa-6-fosfato isomerasa, la proteína ribosomal 50S L5 y la alfa-1,4-glucano-proteína sintasa.

A pesar de los avances en el conocimiento de los mecanismos moleculares de respuesta y resiliencia a la sequía y *P. cinnamomi* generados en la presente Tesis, y considerando la alta variabilidad encontrada en *Q. ilex*, es importante señalar que los mecanismos y marcadores propuestos deben ser validados funcionalmente mediante estrategias de genética inversa, y confirmados para

un mayor número de poblaciones e individuos antes de ser considerado como generales para la especie. El siguiente paso será comprender las bases genéticas y epigenéticas de la resiliencia y las diferencias entre genotipos. En esta dirección, la reciente secuenciación del genoma de la encina llevada a cabo en el grupo es un hito clave.

Chapter I

Introduction and Objectives

1. Introduction

1.1. The genus Quercus

The genus *Quercus* (oak), being the family Fagaceae, comprises around 500 species and is one of the most important woody angiosperms in the Northern Hemisphere in terms of species diversity, ecological dominance and economic value (Figure 1) (Franco, 1990; Gea-Izquierdo *et al.*, 2006; Denk *et al.*, 2017). *Quercus* spp. are dominant of a wide variety of habitats such as temperate deciduous forests, temperate and subtropical evergreen forests, subtropical and tropical savannah and subtropical woodlands (Figure 1).

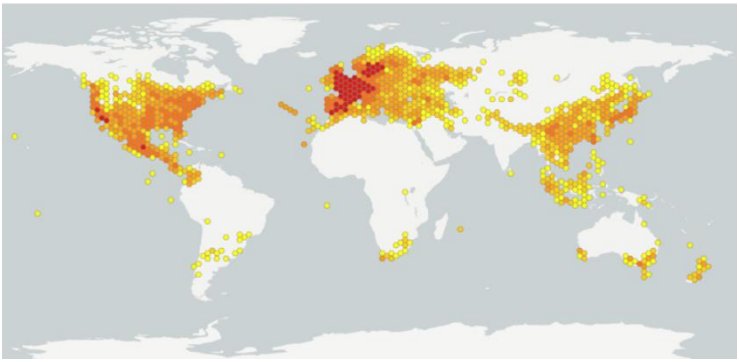

Figure 1. Geographical distribution of species of the genus *Quercus*. The colour scale indicates a higher (red) and a lower (yellow) density of *Quercus* spp. (GBIF Secretriat, 2019; https://www.gbif.org/es/).

In 1753, the genus *Quercus* was catalogued, by Linneo, from a morphological point of view. A total of 14 species were classified as follows: white, red, cerris and ilex oaks. Later, Manos *et al.*, (2001) carried out the first classification based on molecular characteristics by using ITS (internal

transcribed spacers) sequences, distinguishing two subgenera: *Cyclobalanopsis* (Asia) and *Quercus*. Within the latter, species were classified into five sections: *Quercus* with species of great importance at European level (*Q. ilex* or *Q. robur*), *Mesobalanus* (*Q. pyrenaica*), *Cerris* (*Q. suber* o *Q. coccifera*), *Protobalanus* (*Q. chrysolepis*) and *Lobatae* (*Q. rubra*).

This genus has a great economic value due to the production of various products such as cork taken from *Q. suber*, wood for the manufacture of wine barrels from *Q. alba* or the Iberian ham from *Q. ilex*, among others (Oliveira *et al.*, 2002; Cantos *et al.*, 2003; Baca-Bocanegra *et al.*, 2018). Over the centuries, *Quercus* spp. have been related to the human culture due to their use as a source of energy or food included mainly in the Iberian pig diet (Vinha *et al.*, 2016; Johnson *et al.*, 2019; Aung *et al.*, 2020). However, it is remarkable that the preconception of including acorns in the human diet is changing. In a traditional sense, *Quercus* acorns are mainly used in animal feeding, but the interest in integrating acorns into the human diet is raising due to their nutritional value and biological activity (antioxidant, antimicrobial, anticarcinogenic, and cardioprotective properties) (Vinha *et al.*, 2016).

Quercus spp. are of great environmental importance as they form part of different ecosystems being a fundamental pillar of some systems such as "*dehesa*" in Spain, "*montado*" in Portugal, hardwood rangelands in

California, "*terroir*" in France (Standiford *et al.,* 2003). Specifically, the term "*dehesa*" refers to an agrosilvopastoral system (Gil-Pelegrín *et al.,* 2017). The wide distribution of the genus *Quercus* is due to its high plasticity to different edaphoclimatic factors (Valladares *et al.,* 2002; Ramírez-Valiente *et al.,* 2019). Their correct adaptation is based on the development of mechanisms of resistance and tolerance to unfavourable conditions such as high temperatures, prolonged drought, low temperature, or adverse edaphic characteristics. These adaptations eventually lead to phenotypic changes considered as microevolution (Valladares *et al.,* 2002).

1.2. Current issues in the genus Quercus

Currently, the genus *Quercus* is seriously threatened by forest degradation, fragmentation and loss as well as anthropogenic causes (overexploitation, fires, and agricultural land conversions, among others), which are increasing the tree mortality and deforestation (Grantham *et al.,* 2020). Moreover, adverse environmental factors play an important negative role in forest conservation. Such factors, which are termed stresses, are physical, chemical, or biological agents that impose restrictions for the growth and development, and depending on their intensity and duration, they may cause tree death.

Due to the current concern on this genus, scientific production is exponentially increasing (Figure 2). Marañón *et al.,* (2014) evaluated the frequency of words in the most cited articles with the term "*Quercus*" and

found that words such as "Climate-change", "Drought", "Resilience" or "Biodiversity" were clearly highlighted (Figure 2). Moreover, this situation has caused a high concern for the genus *Quercus* by the society as, for example, it happens with Holm oak and *dehesa*. The number of dissemination works about Holm oak in Spain has increased whose main objective of announcing damages caused in this tree species and its environment as well as associated economic costs (https://elpais.com/politica/2018/05/15/actualidad/1526406969_811389.html; https://www.diariocordoba.com/noticias/cordobalocal/junta-destina-30-5-millones-renovar-arbolado-dehesa_1230194.html).

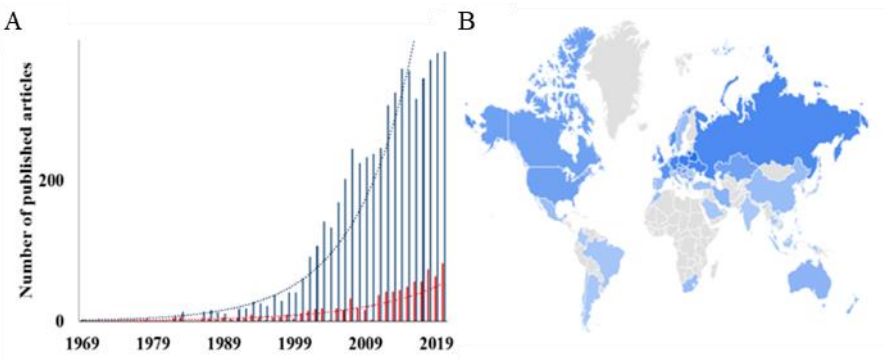

Figure 2. Bibliographic search on the genus *Quercus*. **(A)** Number of scientific works per year found in PubMed from 1969 to 2019 using as keywords "*Quercus*" (dark blue bars) and "Oak decline" (red bars). **(B)** World map showing the interest by the genus *Quercus* in which darker and lighter blue indicate higher lower interest, respectively.

Nowadays, the decline syndrome, affecting the genus *Quercus* and other species, is a good example of threat for forests as well as an international concern. Even, the situation may be aggravated in a climate change scenario (Allen *et al.*, 2010, 2015). Since the 18th and 19th centuries, the decline diseases in oaks are observed in Europe, the Middle East, and North America (Rodríguez-Calcerrada *et al.*, 2017, Denman *et al.*, 2018) (Figure 3). *Quercus* decline is a complex syndrome in which several biotic and abiotic agents interact and, together with inadequate practices, old individuals, and lack of regeneration, lead to a progressive and massive death of trees. The interrelation between abiotic stress (e.g., drought and high temperature) and biotic stress (e.g., pathogens and insects) is complex and there is little information from an experimental point of view.

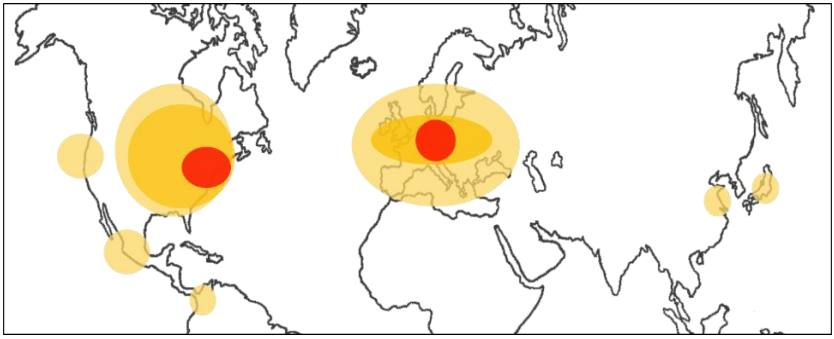

Figure 3. Areas of *Quercus* decline observed since the 18th century. The red colour indicates early reports from the 18th and 19th century and the different shades of yellow colour indicate its expansion between 1900 and 1970 (dark yellow) and after 1980 (light yellow). Image modified from Rodríguez-Calcerrada *et al.* (2017).

The oak decline syndrome is called by different ways depending on the causes and species. In the United Kingdom, the Acute Oak Decline (AOD) syndrome affects both *Q. robur* and *Q. petraea* and is caused by the presence of bacteria (*Gibbsiella quercinecans* and *Brenneria goodwinii* and, to a lesser extent, *Rahnella victoriana* and *Lonsdalea britannica)* and under certain environmental factors (Brown *et al.*, 2016, 2018). On the other hand, in Spain and Portugal, the oak decline syndrome that affects both *Q. ilex* and *Q. suber* is mainly caused by the synergy between *Phytophthora cinnamomi* Rands and long periods of drought (Brasier., 1996; Corcobado *et al.*, 2014; Ruíz-Gómez *et al.*, 2018, 2019). *Phytophthora* spp. is an oomycete considered to be one of the most lethal pathogens described in species of the genus *Quercus* (Brasier, 1996; Gentilesca *et al.*, 2017). Other pathogens affecting these species, such as *Phytium spiculum* Paul and *Biscogniauxia mediterranea* (De Not) Kuntze, are also considered part of this syndrome (Luque *et al.*, 2000; Romero *et al.*, 2007; Ruiz-Gómez *et al.*, 2019). Extreme environmental conditions in a climate change scenario represent a highly relevant issue that will determine species survival as well as forest composition, structure, and functionality (Allen *et al.*, 2010, 2015). Current simulation models predict an increase in both temperature and frequency of severe drought events (Giorgi *et al.*, 2008; Collins *et al.*, 2013), factors that favour the incidence of pathogens such as *P. cinnamomi* and thus the spread of the syndrome to areas where incidence is still low or absent (Hernandez-Lambraño *et al.*, 2018).

1.3. Holm oak as a species of interest

Holm oak (*Quercus ilex* L.), together with cork oak (*Q. suber* L.), are considered a fundamental pillar in the agrosilvopastoral ecosystem "*dehesa*"/ "*montado*" (García-Nogales *et al.*, 2016; Montero, 2017). In total, the estimated surface area of dehesa in the Iberian Peninsula is 3.1 M Ha, with 2.2M Ha corresponding to Spain and 0.9M Ha to Portugal (Díaz Esteban and Pulido Díaz, 2009). According to data published by TRAGSATEC, 2008 (Montero, 2017), the area of dehesa is mainly concentrated in southern Spain (21% in Castilla-La Mancha, 35% in Extremadura and 27% in Andalusia). The dehesa is of great environmental and economic importance and its conservation is a key requirement for the maintenance of the biodiversity of the Mediterranean forest. For this reason, in 1996, dehesas were included as special areas of conservation by the EU Council Directive 92/43/EEC (Council of Europe, UNEP and ECNC, 1996; Mücher and Wascher, 2007).

1.3.1. Holm oak decline syndrome

As it has already been mentioned above, currently, one of the main triggers of the increased rates of death in individuals of Holm oak is the decline syndrome. This syndrome, caused mainly by *P. cinnamomi* and long drought periods, produces root system necrosis, defoliation, and shoot death (Brasier, 1996; Sánchez *et al.*, 2002; Ruiz-Gómez *et al.*, 2018, 2019; Frisullo *et al.*, 2018). To date, few studies have assessed the role of this syndrome (*P. cinnamomi* and drought) on the decline and resilience of Holm oak, although the response to *P. cinnamomi* or drought has been widely assessed

independently (Gea-Izquierdo *et al.*, 2011; Valero-Galván *et al.*, 2013; Sghaier-Hammami *et al.*, 2013; Granda *et al.*, 2013; Camarero *et al.*, 2015; Camarero *et al.*, 2016; Natalini *et al.*, 2016).

Holm oak mature tree is considered as a drought-tolerant species due to several morphological, ecological and physiological adaptations (Barbeta and Peñuelas, 2016; Seleiman *et al.*, 2021). Among the morphological characteristics that allow it to be drought tolerant are its sclerophyllous leaves and a foliage reduction (Schimper, 1903; Peguero-Pina *et al.*, 2014; Gil-Pelegrín *et al.*, 2017). Also, Holm oak trees show a low proportion of fine roots and a relatively high below-ground biomass (Barbeta and Peñuelas, 2016). At physiological level, this forest species for withstanding summer drought closes stomata to reduce xylem tension, low cuticular transpiration and maintenance of cellular turgor by an osmotic adjustment (Barbeta and Peñuelas, 2016). Holm oak, as other evergreen Mediterranean oaks, are considered as water savers due to both lower maximum stomatal conductance and transpiration rates as well as higher stomatal sensitivity to vapour pressure deficit, although the handicap of these species could be their lower photosynthesis rates (Gil-Pelegrín *et al.*, 2017). Nevertheless, although Holm oak shows a high tolerance to drought and high temperature, seedlings are vulnerable to transplanting stress and to summer drought, having high mortality and slow growth compared with other Mediterranean species (Baeza *et al.*, 1991; Villar-Salvador *et al.*, 2004; Ogaya and Peñuelas, 2021).

Phytophthora cinnamomi is a pathogen that mainly attacks the root of the tree, damaging vascular tissues and preventing the absorption of water and nutrients (De Sampaio *et al.,* 2013; Ruiz-Gómez *et al.,* 2014). The pathogenicity of *P. cinnamomi* is classified into two types: either sudden or progressive death. In the former, tree death develops in one or two sessions and appears in early summer after a winter of frequent rainfall. In the second one, firstly, symptoms of chlorosis and necrosis appear with subsequent loss of foliage, decay and, finally, death of tree (De Sampaio *et al.,* 2013). Although the causes of the syndrome are still uncertain, it is known that the attack of *Phytophthora* spp. clearly depends on the physiological state of the tree (Duniway, 1983) needed an external factor, generally abiotic such as drought to cause death to the host (Agrios, 2005).

1.3.2. Biotechnology applied to Holm oak

In view of the worrying situation facing Holm oak, there is an urgent need to propose alternatives for the conservation, sustainable management, reforestation and exploitation of this species and ecosystem. In this regard, Biotechnology is a possibility that could be integrated with other preventive and curative alternatives (cultural, chemical or biological) (Escandón *et al.,* 2021). Biotechnology is the technology based on biological knowledge, especially at the molecular level. In Holm oak, the development of biotechnological approaches is hampered by the limited knowledge of their biology, particularly from a molecular point of view. Different strategies can be performed from a biotechnological point of view such as classical genetic

improvement, genetic engineering, agrochemical searching, and the exploitation of biodiversity. The latter strategy can be considered as the most feasible alternative to be used in Holm oak due to its biology (long biological cycle, non-domesticated and recalcitrant species). Therefore, the exploitation of biodiversity may contribute to the characterization of phenotypic and molecular diversity in this forest species as well as the development of molecular markers related to elite or plus genotypes in terms of higher germination rates, great acorn production with desirable quality traits related to nutritional values, or adaptation to adverse biotic and abiotic stresses (Jorrín-Novo and Navarro-Cerrillo, 2014; Rey *et al.*, 2019; Escandón *et al.*, 2021). To date, the number of scientific works focused on molecular aspects of Holm oak and its responses to environmental factors is quite limited since it is a challenging and difficult task (Abril *et al.*, 2011). In addition, the absence of its sequenced genome complicates studies about its biology.

Our research group has been working on the *Q. ilex* molecular aspects for more than fifteen years, being its main objectives focused on characterize the Holm oak diversity, seed germination and maturation, and responses to single stress (drought or *P. cinnamomi*) from biochemical and modern omic techniques (transcriptomics, proteomics and metabolomics) (Jorge *et al.*, 2005, 2006; Echevarría-Zomeño *et al.*, 2009, 2012; Valero-Galván *et al.*, 2011, 2012, 2013; Sghaier-Hammami *et al.*, 2013, 2016, 2020, 2021; Romero-Rodriguez *et al.*, 2015, 2019; Simova-Stoilova *et al.*, 2015; 2018; Fernández i Marti *et al.*, 2018; López-Hidalgo *et al.*, 2018, 2020, 2021;

Guerrero-Sánchez *et al.,* 2017, 2019; San-Eufrasio *et al.,* 2020, 2021; Escandón *et al.,* 2021).

1.4. Central dogma of molecular biology: Proteomics

The analysis of plants genetic variability can do following the Central Dogma of Molecular Science as, for example, has been recently described in Escandón *et al.* (2021) where *Q. ilex* is taken as example. The Central Dogma of Molecular Biology explains the flow of genetic information in a biological system starting with DNA that is copied to DNA (DNA replication) or into RNA (transcription). Then, proteins are synthesized using the RNA as a template (translation) (Crick, 1970).

Proteomics has had a big impact on plant biology, considered as a valuable tool for several forest species, such as *Quercus*, *Pines*, *Poplars*, and *Eucalyptus* (Rey *et al.,* 2019). It has proven their value in assessing genetic changes in some plant species (Bahrman *et al.,* 1995; Emre *et al.,* 2009; Jorrín-Novo *et al.,* 2009; Abril *et al.,* 2011) as well as in characterizing the natural variability of Holm oak (Valero-Galván *et al.,* 2011, 2012). Proteomics is a leading technology for the high-throughput analyses of proteins on a genome-wide scale that with both genome sequencing and protein characterization by using analytical methods, this approach has become a major field of functional genomics (Park *et al.,* 2004; Jorrín-Novo, 2021). It can give a specific identification through the evaluation of the protein profile by using one-dimensional (1D) electrophoresis techniques, as

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis), which separate proteins by its molecular weight; two-dimensional electrophoresis techniques (2D), which separate proteins by their isoelectric point and molecular weight; and shotgun-mass spectrometric analysis, using a combination of liquid chromatography coupled to mass spectrometry (LC-MSMS), which is based on the peptides identification by means of its relative mass-to-charge ratio (m/z). Therefore, Proteomics provides a wealth of information that can be complemented with the use of other omics techniques, such as genomics, transcriptomics, metabolomics or epigenomics as well as biochemical and physiological approaches in the direction of Systems Biology (López-Hidalgo *et al.,* 2018; Rey *et al.,* 2019; Escandón *et al.,* 2021).

Focusing on the decline syndrome, so far, the response both to drought and *P. cinnamomi* have been determined independently in Holm oak seedlings by a proteomic approach (Jorge *et al.,* 2006; Echevarría-Zomeño *et al.,* 2009; Valero-Galván *et al.,* 2013; Sghaier-Hammami *et al.,* 2013; Simova-Stoilova *et al.,* 2015, 2018). Changes in the leaf protein profile caused by the attack of *P. cinnamomi* have been described in two Andalusian *Q. ilex* populations (Cordoba and Almeria) by using a 2-DE coupled to MS proteomics strategy (Sghaier-Hammami *et al.,* 2013). The response to this biotic stress was carried out mainly by chloroplast proteins involved in the photosynthesis, Calvin cycle and carbohydrate metabolism. In this study was remarkable that the protein abundance was lower in the inoculated seedlings than in the non-

inoculated seedlings. These changes in protein abundance have been frequently observed in plants subjected to drought stress. Regarding the effect of drought in Holm oak, several studies have been performed in seedling leaves by using 2-DE electrophoresis and mass spectrometry analysis (Jorge *et al.,* 2006; Echevarría-Zomeño *et al.,* 2009; Valero-Galván *et al.,* 2013; Simova-Stoilova *et al.,* 2015, 2018). The drought conditions caused changes in the profile of proteins belong to the photosynthesis, carbohydrate and nitrogen metabolism, and stress (Echevarría-Zomeño *et al.,* 2009; Valero-Galván *et al.,* 2013). As with the attack of *P. cinnamomi*, a decrease in protein abundance upon water withholding was described in these studies.

2. Objectives

2.1. General objective

The aim of this work was to to go deeper into the knowledge of the biological variability present in *Quercus ilex* in the response to stresses associated to the decline syndrome, drought and *Phytophthora cinnamomi,* to understand the mechanisms of resilience and to identify molecular markers to be used in breeding programs.

2.2. Specific objectives

1. Evaluate the response and differences in tolerance to drought in seedlings from *Quercus* spp. and Andalusian *Q. ilex* populations *(Chapter II*). The response to drought will be conducted in seedlings of five *Quercus* spp. (*Q. ilex*, *Q. suber*, *Q. pyrenaica*, *Q. robur* and *Q. faginea*) and five Andalusian *Q. ilex* populations (Cadiz, Seville, Cordoba, Granada and Jaen) grown in perlite under severe drought conditions representative of the summer conditions of southern Spain. Drought tolerant individuals within species variability will be identified and characterized from a physiological and biochemical point of view.

2. Identify proteins and derived proteotypic peptides to be proposed as putative drought tolerance markers by proteomics data analysis *(Chapter III)*. A double strategy will be used to quantify proteins and peptides by targeted post-acquisition data analysis against a species-specific *Q. ilex* database to identify changes in proteins and derived peptides

persistent over time in response to drought in four Andalusian *Q. ilex* populations (Cadiz, Seville, Huelva and Granada).

3. Determine the response to drought and *Phytophthora cinnamomi* in three Andalusian *Q. ilex* populations by using physiological and proteomic approaches *(Chapter IV).* The response and tolerance mechanisms to *P. cinnamomi* and drought will be carried out in three contrasted Andalusian *Q. ilex* populations (Seville, Granada and Almeria), single and combined, from a physiological, biochemistry and proteomic point of view.

Chapter II

Responses and differences in tolerance to water shortage under climatic dryness conditions in seedlings from *Quercus* spp., and Andalusian *Q. ilex* populations

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Abstract

Analysing differences in tolerance to drought in *Quercus* spp., and the characterization of these responses at the species and individual population level are imperative for the selection of resilient elite genotypes in reforestation programmes. The main objective of this work was to evaluate differences in the response and tolerance to water shortage under in five *Quercus* spp., and five Andalusian *Q. ilex* populations at the inter- and intraspecies level. Six-month-old seedlings grown in perlite were subjected to drought treatments by withholding water for 28 days under mean 37 ºC temperature, 28 W m-2 solar irradiance, and 41% humidity. The use of perlite as the substrate enabled the establishment of severe drought stress with reduction in water availability from 73% (field capacity) to 28% (dryness), corresponding to matric potentials of 0 and -30 kPa. Damage symptoms, mortality rate, leaf water content, photosynthetic, and biochemical parameters (amino acids, sugars, phenolics, and pigments) were determined. At the phenotypic level, based on damage symptoms and mortality, *Q. ilex* behaved as the most drought tolerant species. Drought caused a significant decrease in leaf fluorescence, photosynthesis rate and stomatal conductance in all *Quercus* spp. analysed, being less pronounced in *Q. ilex*. There were not differences between irrigated and non-irrigated *Q. ilex* seedlings in the content of sugar and photosynthetic pigments, while the total amino acid and phenolic content significantly increased under drought conditions. As a response to drought, living *Q. ilex* seedlings adjust stomata

opening and gas exchange, keep hydrated, photosynthetically active, and metabolically competent. At the population level, based on damage symptoms, mortality, and physiological parameters, the eastern Andalusian populations were more tolerant than the western ones. These observations propose the bases for the selection of resilient genotypes to be used in breeding and reforestation programs.

Keywords: *Quercus* spp.; *Quercus ilex*; drought; climate change; abiotic stress; biodiversity; perlite

1. Introduction

The genus *Quercus* comprises around 300 species, 25 of which are native in Europe, and 9 in the Iberian Peninsula (Franco, 1990). *Quercus* spp. are distributed in a wide variety of habitats such as temperate and subtropical forests, varying from wet to extremely dry habitats (Nixon, 2006). Within this genus, *Q. ilex* constitutes the most representative species in the Mediterranean forest and agrosilvopastoral ecosystem "*dehesa*" with a high environmental and economic relevance (Moreno and Pulido, 2009). However, the current situation of the species is worrying due to increased tree mortality observed in the last decades (Natalini *et al.,* 2016). Tree mortality is associated with several factors of an anthropogenic or natural origin, most pertinent being biotic and abiotic stresses (Plieninger, 2007; Surová *et al.,* 2018; Ruíz-Gómez *et al.,* 2019). Although Holm oak (*Q. ilex*) is considered a species that is well-adapted to a dry climate (Echevarría-Zomeño *et al.,* 2009; Valero-Galván *et al.,* 2013; Gil-Pelegrín *et al.,* 2017), drought stress is the main cause of *Q. ilex* seedling mortality in forest plantations, especially in Andalusia, where this stress is a limiting factor in its regeneration (Leiva and Fernández-Alés, 1998; Villar-Salvador *et al.,* 2004; Navarro-Cerrillo *et al.,* 2005; Forner *et al.,* 2018). Moreover, the situation could become even worse in a climate change scenario (Lloret *et al.,* 2004; Natalini *et al.,* 2016), in which simulation models predict an increase both in temperature and frequency of severe drought episodes (Giorgi and Lionello 2008; Collins *et al.,* 2013).

Plant responses to drought stress at the physiological and molecular levels have been widely studied in a wide variety of forest species (David *et al.*, 2007; Peng *et al.*, 2012; Früchtenicht *et al.*, 2018). Some studies have been carried out to determine the response to drought in some species of the genus *Quercus* from a morphological and physiological point of view (Hamerlynck and Knapp, 1996; Vilagrosa *et al.*, 2012; Forner *et al.*, 2018). Under drought conditions, species of the genus *Quercus* have developed drought-tolerance mechanisms such as a deep and well-structured root system to maintain a relatively high predawn potential, resistance of the xylem to cavitation and embolism, and stomatal closure (Vander Mijnsbrugge *et al.*, 2010; North *et al.*, 2019). The acorn size and reserve nutrients are also related to the survival and growth under adverse conditions. Quero *et al.* (2007) reported beneficial effects of larger seed in *Quercus* seedlings, such as seedling establishment in nutrient poor soils and longer roots that favor the survival of seedlings in the first summer drought. Regarding acorn reserve nutrients, Villar-Salvador *et al.*, (2010) revealed that most of the nitrogen (N) accumulated in holm oak seedlings came from the acorn at the end of the second shoot flush of growth (three months after shoot emergence), being only 25-38% of the N taken up by the roots. In contrast, acorn N reserved in *Q. robur* are reduced at the end of the first shoot flush of growth (García-Cebrián *et al.*, 2003).

Studies with Mediterranean *Quercus* species indicate that genetic variation may have contributed to the large differences in drought tolerance and within

populations (Arend *et al.,* 2011; Navarro-Cerrillo *et al.,* 2018). Ramírez-Valiente *et al.* (2009) reported a high degree of inter- and intrapopulation variability for traits related to drought tolerance, and Arend *et al.* (2011) showed similar results in *Q. robur*, *Q. petraea* and *Q. pubescens.* However, the selection of drought tolerant individuals based on the intraspecies variability is still limited. For instance, the identification and characterization of plus or elite *Q. ilex* genotypes, which are more resilient and tolerant to drought, is a priority in plant breeding, reforestation, management, and conservation programmes in the Mediterranean area. To enable this, ecophysiological and molecular studies are required to have a better understanding of both the variability in this species and the selection of morphometric, physiological, and molecular markers (Rey *et al.*, 2019).

Thus, in this study, we have evaluated the effect of drought stress in six-month-old seedlings to determine differences in the response and tolerance to this stress in a total of five species of the genus *Quercus*, and five Andalusian *Q. ilex* populations. Six-month-old seedlings were selected due to the negative impact of first summer drought in the seedling mortality. Differences from previous published studies are fourfold: i) the experiments were conducted under severe drought conditions representative of the summer conditions of southern Spain, and those predicted in a climate change scenario, characterized by high temperatures, radiation and low humidity; ii) the use of seedlings grown in perlite which enables the

imposition of a rapid and severe drought stress; iii) the simultaneous comparison, in a single experiment, of five *Quercus* spp. and five Andalusian *Q. ilex* populations; and iv) the identification and characterization of drought tolerant individuals within species variability from a physiological and biochemical point of view.

2. Materials and Methods

2.1. Study Area

Drought experiment was conducted on five *Quercus* spp. (*Q. pyrenaica*, *Q. faginea*, *Q. robur*, *Q. suber*, and *Q. ilex*) and five *Q. ilex* populations from Andalusia (Gr: Arenas del Rey (Granada); Ja: Sierra de Segura (Jaen); Se: Almaden de la Plata (Seville); Co: Pozoblanco (Cordoba) and Ca: Behamahoma (Cadiz)) in Cordoba, Andalusia, Southern Spain at 37°54′46″N, 4°43′15″O in July, 2018 for 28 days. Cordoba is under Mediterranean climate with 36ºC and 19ºC maximum and minimum temperatures, respectively, 28 W m-2 solar irradiance, and 42% relative humidity in July over the period 2005-2019.

Table 1. Environmental features of the *Quercus* spp. and Andalusian *Q. ilex* populations used. Altitude (Meters Above Sea Level-MASL), average temperature of the coldest month (T_{min}), average temperature of the warmest month (T_{max}), and average annual rainfall (P) (Navarro-Cerrillo *et al.*, 2018). ES code indicates the region of origin as detailed in "*Centro Nacional de Recursos Genéticos Forestales, El Serranillo"*). Location of all species is included in Figure S1.

Species	Location	MASL (m)	T_{max} (ºC)	T_{min} (ºC)	P (mm)
Quercus robur	ES01	488	24.8	2.2	1375
Quercus faginea	ES10	1032	27.6	-0.1	577
Quercus pyrenaica	ES08	823	24.5	-1.8	792
Quercus suber	ES01	456	33.7	2.4	824
Quercus ilex	ES11	506	34.5	2.6	635
Quercus ilex (Jaen)	38°17´N, 2°36´W	643	23.1	4.4	795
Quercus ilex (Granada)	36°57´N, 3°54´W	489	24.7	11.5	489
Quercus ilex (Cordoba)	38°22´N, 4°54´W	618	26.8	8.1	613
Quercus ilex (Cadiz)	36°45´N, 5°27´W	649	24.9	9.8	1264
Quercus ilex (Seville)	37°52´N, 6°05´W	482	26.4	9.5	722

2.2. Plant material, growth conditions, and drought treatment

Acorns from different *Quercus* spp., and Andalusian *Q. ilex* populations were used. The populations were chosen according to Valero-Galván *et al.* (2013) and Fernández i Marti *et al.* (2018). Healthy acorns were selected, germinated, and sown leaving acorns in the development of seedlings, in black plastic pots (3L, 14.5 × 14.5 x 22 cm) containing perlite (Gramoflor GmbH and Co. KG Diepholzer Strabe 173, Vechta, Germany), and grown in a greenhouse as previously reported Simova-Stoilova *et al.* (2015). All the

seedlings were germinated in January 2018 and irrigated every two days with approximately 200 ml of tap water per pot and once a week with a Hoagland nutrient solution (Hoagland, 1950). To achieve a severe and rapid drought stress, perlite was used as the substrate, an alumino-silicate of volcanic origins characterized by a closed cellular structure. Severe drought was imposed by withholding water for 28 days under the following environmental conditions: mean values of 46ºC and 22ºC maximum and minimum temperatures, 28 MJ m^{-2} per day^{-1} solar irradiance, and 41% relative humidity. The experiment was performed based on a completely randomized design with ten biological replicates per treatment to analyse damage symptoms and mortality rate caused by drought (in total 200 seedlings). Out these biological replicates, three individuals were randomly selected for measuring relative leaf water content, physiological parameters, or biochemical analyses (in total 60 seedlings). *Quercus ilex* was considered as the mean of all the Andalusian populations analysed.

2.3. Perlite water content and matric potential

Perlite water content (PWC) was estimated by weighing pots containing dry or totally wet (field capacity) perlite as well as the pots corresponding to the different days of the experiment. The following formula was employed:

$$PWC_t\ (\%) = (\text{pot wet weight}_t - \text{pot dry weight}/\text{pot wet weight}_0 - \text{pot dry weight}) \times 100$$

where t corresponds to the different days, and 0 to the initial, maximum value.

Perlite matric potential (Ψ_m, kPa) was measured by using a tensiometer (Soil Moisture Equipment corp, Santa Barbara, CA, USA) at 15-cm depths. Both parameters were plotted, and a correlation equation obtained.

2.4. Damage symptoms and seedling mortality

Damage symptoms (leaf chlorosis, wilting, and senescence) caused by the drought treatment was visually observed throughout the whole experiment. Photographs were taken with a digital camera to register damages. Visual damage symptoms were quantified every three days according to a 0-5 scale where 0 = no leaves showing symptoms; 1 = one or two leaves showing slight drought symptoms (necroses along edges and/or veins; changes in the colour of foliage (light green, yellow and brown); and/or irregular spot changes); 2 = most leaves showed slight levels of drought symptoms however one or two leaves still showed no symptoms; 3 = all leaves showed drought symptoms but these were not severe; 4 = all leaves showed severe drought symptoms (leaves showed a totally dry-yellow aspect); and 5 = the whole seedling showed wilting and/or fall of leaves. The number of dead seedlings was also recorded when they were classified in 4-5 scale and Fv/Fm values near 0.

2.5. Leaf water status

Relative leaf water content (RLWC) was calculated at day 25 from fresh (FW), turgid (TW) and dry (DW) weights. Upon leaf removal (three living seedlings per treatment and one non-damaged leaf per seedling), FW was determined, then leaves were soaked in distilled water at 25ºC for 24 h and weighted again to record the TW. For dry weight measurements, leaves were kept in an oven at 65ºC for five days until weight remained constant. RLWC was calculated according to the formulae:

$$RLWC\ (\%) = [(FW - DW)/(TW - DW)] \times 100.$$

2.6. Quantum yield of photosystem II, net photosynthesis rate and stomatal conductance

Leaf fluorescence and derived photochemical efficiency of photosystem II (F_v/F_m) in dark-adapted seedlings was measured regularly with a portable fluorometer (FluorPen FP100, Photon Systems Instruments, Drásov, Czech Republic) (Bilger *et al.*, 1995). Three values per seedling in the youngest fully expanded leaves were taken every three days in the early morning, when the leaves were adapted to darkness throughout the night according to Strasser *et al.* (2000). All measurements were carried out in the same seedlings throughout the experiment. The net photosynthesis rate (*A*, µmol CO_2 m^{-2} s^{-1}) and stomatal conductance (*Gs*, mol H_2O m^{-2} s^{-1}) were measured in three fully-expanded leaves in the *Quercus* spp., at day 9, and in Andalusian *Q. ilex* populations at day 21 by using a portable infrared CO_2

gas analyzer (LiCor Li6400XT, Li-Cor, Inc.; Lincoln, NE, USA) fitted with a 6 cm^2 leaf cuvette. The measurements were taken using a CO_2 concentration of 400 ± 1.7 ppm, a flow of 300 ± 1.2 cm^3 min^{-1}, and PPFD >1000 mol (photons) m^{-2} s^{-1}. All the measurements of physiological variables were taken between 12:00–14:00 h UTC (Universal Time Coordinates), considering a 2 h window around the solar noon (12:00–14:00 h CET—Central European Time) (Ruíz-Gómez *et al.*, 2019).

2.7. Photosynthetic pigment and anthocyanin analyses

Leaves from the *Q. ilex* Se population (three biological replicates) were used to quantify the total content of chlorophylls (a and b), carotenoids, and anthocyanins according to Sims and Gamon (2002), at day 25 of the experiment. Briefly, they were extracted by using two solutions, the first being acetone:Tris (1M) pH 7.8 (80:20 v/v) and the second methanol:1% HCl:water (90:1:1, v/v/v)). Supernatants, after homogenization and centrifugation, were used to read absorbance at 663, 647, 537, and 470 nm (Thermo Scientific Evolution 201 UV-Visible Spectrophotometers), from which the pigment and anthocyanin contents were calculated.

2.8. Total sugar, phenolic, and amino acid analysis

Leaves from seedlings of the *Q. ilex* Se population (three biological replicates) were used to quantify total sugar, phenolic, and amino acid content at day 25 of the experiment, by using, respectively, 3, 5-

dinitrosalicylic acid (DNS) (Miller, 1953), Folin-Ciocalteu (Ainsworth and Gillespie, 2007) and ninhydrin (Starcher, 2001) methods. Briefly, all samples were extracted using chloroform/methanol/water (1:2,5:0.5, v/v/v). Reference standards of glucose, chlorogenic acid and glycine were used for quantitation, respectively. Supernatants, after homogenization and centrifugation, were used to read absorbance at 570 nm (sugars and amino acids) and 765 nm (phenolics) (Thermo Scientific Evolution 201 UV-Visible Spectrophotometers).

2.9. Statistical analyses

All the statistical analyses were performed using STATISTIX 10.0 software (Analytical Software, Tallahassee, FL, USA). The following statistical tests were employed: Student t ($p<0.05$) was used for total phenolic, sugar, amino acid, and pigment values and for the photosynthesis rate and stomatal conductance data. When the homogeneity of variance was not satisfied, the Kruskal Wallis´s test was used; Kruskal Wallis ($p<0.05$ and means separated by the Dunn test at $p<0.05$) for damage symptoms; One-way ANOVA ($p<0.05$ and means separated by the *post hoc* least significant difference, LSD, test at $p<0.05$) for F_v/F_m and photosynthesis rate and stomatal conductance parameters in non-irrigated seedlings at species and population levels. Both in the Kruskal Wallis and the one-way ANOVA tests, the area under the curve for damage symptoms and F_v/F_m was calculated. Two-way ANOVA ($p<0.05$ and means were separated by the LSD test at $p<0.05$),

using species/populations and treatment as factors, for RLWC. The Levene´s and Folded F tests were used to determine the homoscedasticity of the variables for ANOVA and t-test, respectively.

3. Results

3.1. Perlite water content and matric potential

Perlite water content (PWC) percentages decreased from 73% (field capacity) at zero time to 28% at day 28, which corresponded to matric potentials, as determined by tensiometry, of, respectively, 0 and -30 kPa. A linear regression was obtained for both parameters with equation

$$\Psi_m \text{ (kPa)} = -51 + 0.68 \text{ PWC (\%)} \ (R^2 = 0.981)$$

Drought caused a linear decrease in perlite water content, with experimental data fitting in the following equation:

$$\text{PWC (\%)} = (0.71 - 0.015 \text{ days}) \times 100 \ (R^2 = 0.992)$$

or

$$\Psi_m \text{ (kPa)} = 1.48 - 1.20 \text{ days} \ (R^2 = 0.990)$$

3.2. Evaluation of damage symptoms and seedling mortality

Neither chlorosis nor wilting was observed in irrigated seedlings. Leaf damage symptoms occurred to differing degrees in non-irrigated seedlings depending on the surveyed species and populations. The first *Quercus* species displaying visible stress symptoms in the foliage were *Q. faginea* and *Q. pyrenaica*, beginning with yellow-brown necrosis at the edges and tips of the youngest fully expanded leaves, followed by leaf wilting and fall. *Quercus robur* displayed an intermediate behaviour, while *Q. ilex* and *Q. suber* had both individuals with slight (most leaves showed drought symptoms but these were not severe) or serious (all leaves showed a totally dry-yellow aspect and wilting) damage symptoms at the end of the experiment (Figure 1A, Figure S2). At day 12 (57% PWC and -13 kPa Ψ_m values), 100% mortality was recorded for *Q. faginea* and *Q. pyrenaica*, and at day 28 (28% PWC and -32 kPa Ψ_m values), *Q. robur* had 100% and, *Q. suber* and *Q. ilex* 40% mortality rates. In the Andalusian *Q. ilex* populations analysed, from seedlings without symptoms to seedlings with all leaves showed a totally dry-yellow aspect and wilting were observed in leaf symptoms, and the number of dead seedlings observed varied between 40 and 80% in Ja and Ca populations, respectively, at day 28 (Figure 1B).

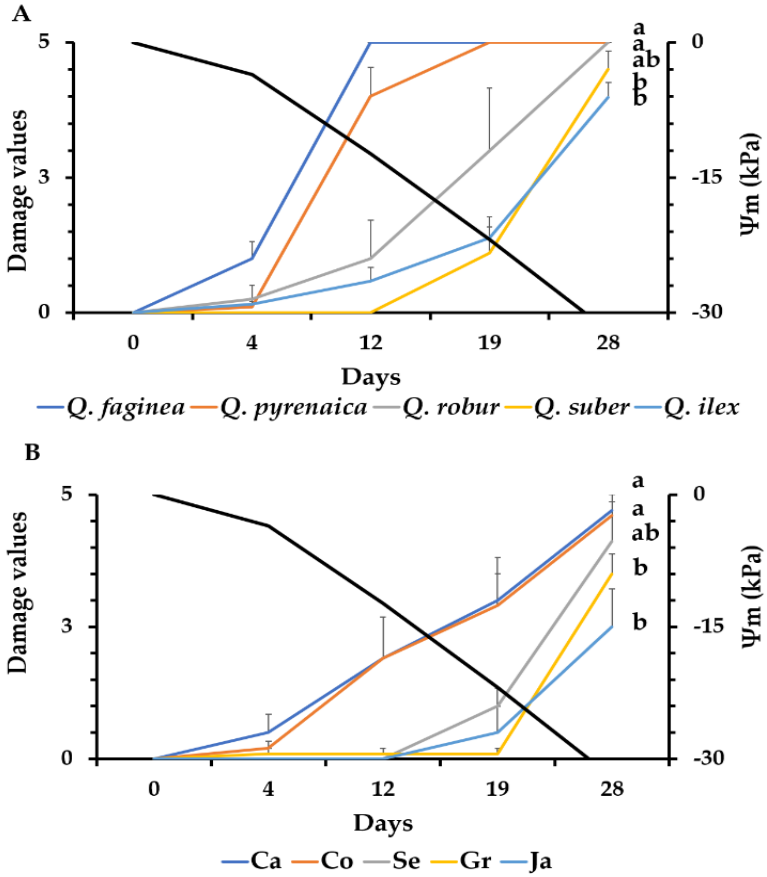

Figure 1. Damage symptoms in drought-stresses seedlings of *Quercus* spp. **(A)** and *Q. ilex* Andalusian population **(B)** throughout the experiment. Symptoms were quantified in a 0-5 scale based on visual evaluation (see material and methods section). Data are mean of ten biological replicates ± SE. The same letter indicates that there is no statistical difference between populations ($p=0.05$). The black line corresponds to the theoretical Ψ_m values (Ψ_m (kPa) = -51 + 0.68 PWC (%)).

3.3. Relative water content in Q. ilex leaves

Mean values in the irrigated and non-irrigated seedlings ranged between 76.6 – 86.3% and 43.1 – 70.7%, respectively (Figure 2). Drought caused a statistically significant reduction in RLWC (*F=51.93; p=0.0000*), with differences among populations (*F=3.38; p=0.0313*) (Figure 2). Under drought conditions, Gr and Ca populations showed the highest and lowest RLWC.

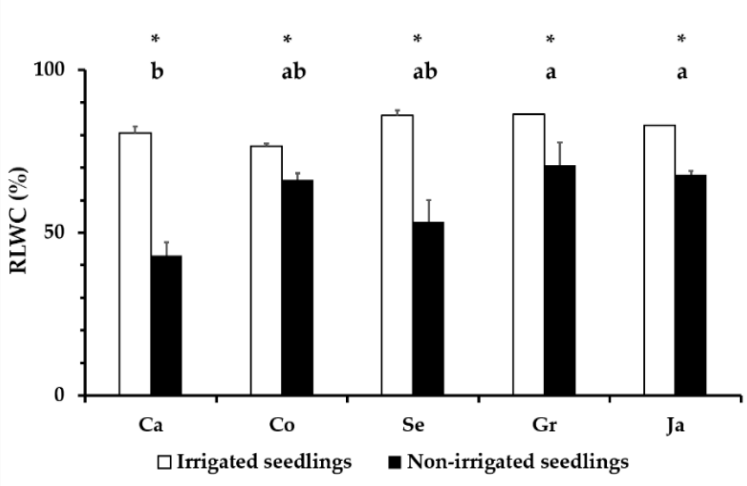

Figure 2. RLWC determined in seedlings of the Andalusian *Q. ilex* populations at day 25. Values are mean ± SE of three biological replicates. Statistically significant differences were observed between irrigated and non-irrigated seedlings. Different letter indicates that there is significant difference among populations (*p<0.05)*. Asterisk indicates significant differences between irrigated and non-irrigated seedlings (*p<0.05*).

3.4. Quantum yield of photosystem II (F_v/F_m)

Leaf fluorescence and derived PS II quantum yield at dark-adapted state (F_v/F_m) remained nearly constant (between 0.60 and 0.80) throughout the experiment in irrigated seedlings both in *Quercus* spp., and Andalusian *Q. ilex* populations (Figure S3).

In non-irrigated seedlings, the general tendency observed was a progressive reduction in F_v/F_m values throughout the drought period (Figure 3). This occurred first in *Q. faginea* and then in *Q. pyrenaica* which both showed a sudden and rapid decline to zero values at day 6 (PWC and Ψ_m values of 66% and -6 kPa, respectively), and 10 (PWC and Ψ_m values of 60% and -10 kPa, respectively), respectively (Figure 3A). A less pronounced and slower decrease was observed for *Q. robur* and *Q. suber,* with values of 0.15 and 0.18 determined at day 27, respectively (PWC and Ψ_m values of 29% and -31 kPa, respectively). In the case of *Q. ilex*, significant differences were observed between irrigated and non-irrigated seedlings (*F=0.48; p=0.0007*); however, no significant differences were observed between populations (*F=0.65; p=0.9986*). *Quercus ilex* showed the highest F_v/F_m values in non-irrigated seedlings, with values in the range of 0.31-0.49 measured at day 27 (PWC and Ψ_m values of 29% and -31 kPa, respectively), and depending on the population, with the lowest and highest values corresponding to the Ca and Ja populations, respectively (Figure 3B).

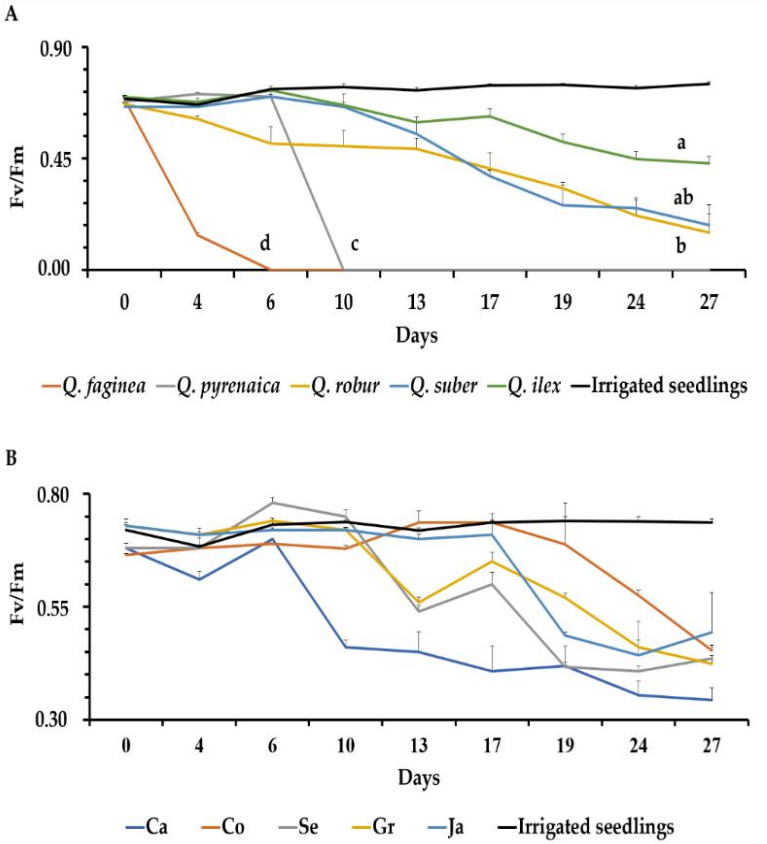

Figure 3. Measurements of quantum yield of photosystem II (F_v/F_m) in dark adapted leaves from *Quercus* spp. **(A)**, and *Q. ilex* interpopulation species **(B)** (non-irrigated seedlings) during drought progression. Values are mean ± SE of three biological replicates. In *Quercus* spp., the same letter indicates no significant difference between species ($p<0.05$). The black line indicates the mean values of irrigated seedlings shown in Figure S3. The lack of letters in Andalusian *Q. ilex* populations indicates no significant differences between populations.

3.5. Leaf Photosynthesis Parameters

Living *Q. ilex* non-irrigated seedlings showed higher *A* and *Gs* values than the rest of the species of the genus *Quercus* (Figure 4A and B). Significant differences in *A* and *Gs* were observed between irrigated and non-irrigated seedlings in all the species analysed, except *Q. ilex*, with significant differences for *Gs* (Figure 4) but none for *A*. As for Andalusian *Q. ilex* populations, at day 9, significant differences were observed between treatments in the Co and Ja populations in *A* and in the Co, Gr, and Ja populations in *Gs* (Figure 4C and D). Under drought conditions, Se and Ca populations showed the highest values in *A* and *Gs*, respectively (Figure 4C and D). At day 21, drought caused a decrease in *A* and *Gs* values in all populations (Figure 4D and E). In *A* and *Gs*, under drought conditions, Ca showed the highest values in both parameters, and Se gave the lowest *A* values of the populations (Figure 4D and E).

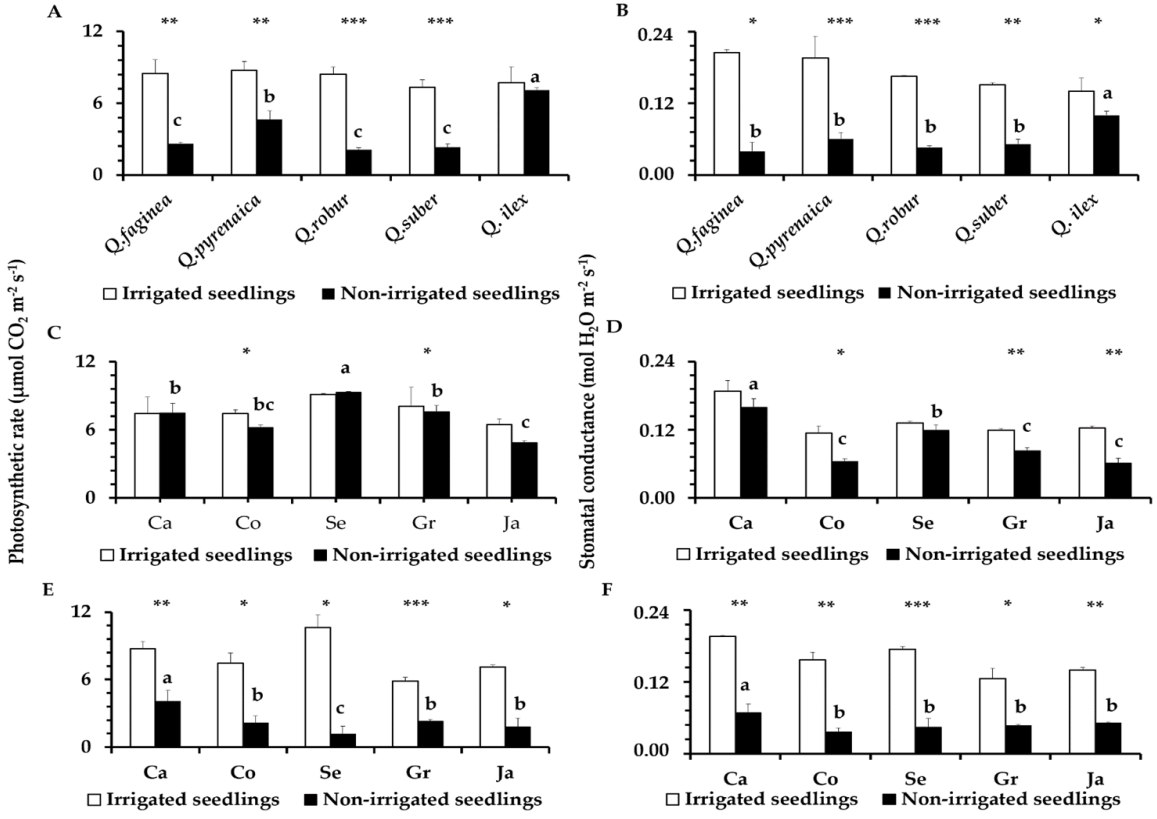
A
B
C
D
E
F
Photosynthetic rate (μmol CO_2 m^{-2} s^{-1})
Stomatal conductance (mol H_2O m^{-2} s^{-1})
Q.faginea
Q.pyrenaica
Q.robur
Q.suber
Q. ilex
Ca
Co
Se
Gr
Ja
Irrigated seedlings
Non-irrigated seedlings

Figure 4. Photosynthesis rate, *A*, and stomatal conductance, *Gs*, in seedlings of the surveyed *Quercus* spp. and Andalusian *Q. ilex* populations, taken at day 9 (species (A and B, respectively) and populations (C and D, respectively) and day 21 (populations (E and F, respectively)). Values are mean ± SE of three biological replicates. Asterisk indicates significant differences between treatments ($p<0.05$ (*); 0.01 (**); 0.001(***)). In the non-irrigated seedlings, the same letter indicates that there is no significant difference between species or populations ($p<0.05$).

3.6. Pigment content and anthocyanins analysis

Figure 5A shows both photosynthetic pigment and anthocyanin content in *Q. ilex* Se seedlings at day 25 under drought conditions. No significant differences were observed between irrigated and non-irrigated seedlings in photosynthetic pigments, with mean value ranges being 0.21-0.22 µmol/g (chlorophyll a), 0.06-0.07 µmol/g (chlorophyll b), and 0.13-0.15 µmol/g (carotenoids). Anthocyanin content was significantly lower in the non-irrigated than the irrigated seedlings, with mean values of 0.07 and 0.09 µmol/g, respectively ($T=3.50$; $p=0.0249$) (Figure 5A).

3.7. Total sugar, phenolic, and amino acid analysis

Figure 5B shows the primary metabolism in *Q. ilex* Se seedlings at day 25 under drought conditions. Mean values ranged between 4.21 and 7.90 µmol/g dry weight (amino acids), 33.14 and 37.29 µmol/g dry weight (phenolics), and 84.66 and 131.19 µmol/g dry weight (sugars). Drought caused a significant increase in the three parameters (Figure 5B, $T=-2.92$; $p=0.0434$, $T=-3.73$; $p=0.0202$, and $T=-3.74$; $p=0.0201$, respectively).

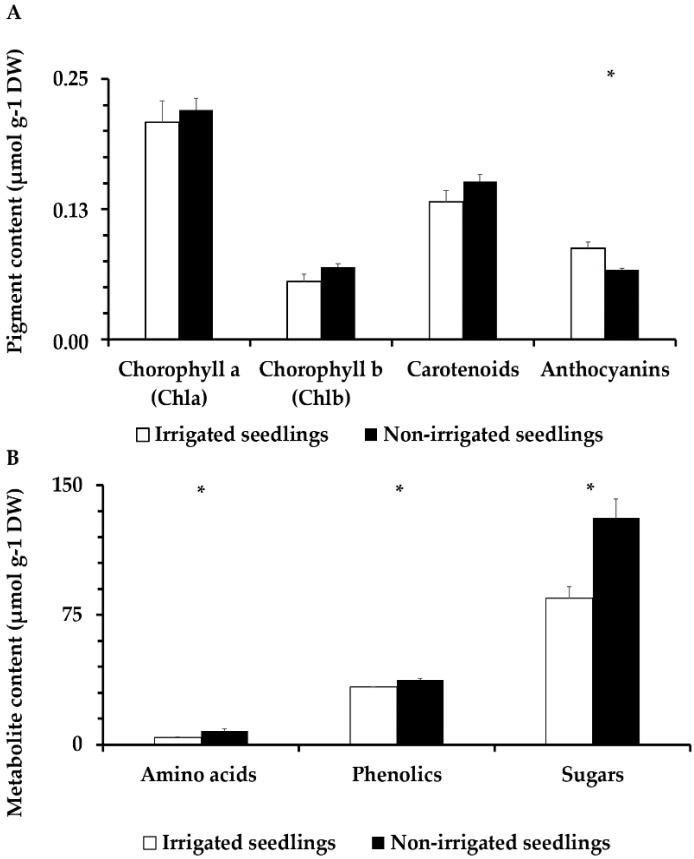

Figure 5. Photosynthesis pigment (chlorophyll (a and b) and carotenoids) and anthocyanins **(A)** and metabolite (phenolics, sugars, amino acids) **(B)** contents in leaves of the *Q. ilex* Se population. Values are mean ± SE of three biological replicates at day 25. Asterisk indicates significant differences between irrigated and non-irrigated seedlings ($p<0.05$ (*)).

4. Discussion

By utilising a successful germination procedure (Simova-Stoilova *et al.,* 2015) with an innovative use of perlite under typical summer conditions reached in Southern Spain, a quick and severe drought stress was achieved in this study. The imposition of these stress conditions in a reasonable experimental time (1 month) is necessary for species that like *Q. ilex* are highly tolerant, and essential for subsequent molecular analysis.

Reduced soil water content and reduced matric potential were directly associated with plant drought stress; factors associated with plant growth (Jensen *et al.,* 1998; Mantovani *et al.,* 2013). Aliniaeifard *et al.* (2010) reported better vegetative and physiological characteristics (root fresh weight, root dry weight, leaf number shoot:root ratio, among others) in perlite compared to a sandy loam farm soil. In another study, a comparison of several substrates (tuff, sand, peat moss, lightweight expandable clay aggregate (LECA), cocopeat) determined that tuff, LECA and perlite have a higher ability to tolerate drought (Tala *et al.,* 2020). Perlite retains most water superficially, and releases it slowly at a relatively low tension, which therefore requires frequent irrigation to prevent a fast-developing water stress (Maloupa *et al.,* 1992). Thus, in our experiments, water content decreased from 73% (field capacity) at day 0 to 28% at day 28, corresponding to matric potentials of, respectively, 0 and -30 kPa, with high correlation between both parameters (Ψ_m (kPa) = -51 + 0.68 PWC (%) (R^2 = 0.981)). Moreover, this substrate could be used as an alternative to hydroponics as it may mimic a higher mechanical impedance to root growth and allow a higher

control of water and nutrient conditions than in soil (Sudhakar *et al.*, 2016). This substrate is more commonly used in herbaceous and horticultural species (barley (Hanson *et al.*, 1977), gerbera (Syros *et al.*, 2004), and tomato (Al-Shammari *et al.*, 2018)), and in other woody (citrus (Arbona *et al.*, 2005; Manzi *et al.*, 2015) and shrub species (rose (Samartzidis *et al.*, 2005)). However, we show that perlite could be also considered as a useful substrate to induce a drought stress in forest trees, as it enables good control of treatments (Jones, 2007).

4.1. Drought visual symptoms and mortality rate as indicators of drought tolerance

Both leaf damage and seedling death were higher and more rapid in the deciduous oak species (*Q. pyrenaica*, *Q. faginea* and *Q. robur*) than in the evergreen oak species (*Q. suber* and *Q. ilex*), as previously reported by (Gil-Pelegrín *et al.*, 2017). Both leaf morphology and structure may be related to different mechanisms to cope with tolerance to drought. Previous studies have demonstrated that the reduction in leaf size is associated with dry habitats (Corcuera *et al.*, 2002; Peguero-Pina *et al.*, 2014). This fact could explain the delay in leaf damages and lower number of dead seedlings observed in *Q. ilex* and *Q. suber* since both species have the smallest leaf sizes of all *Quercus* spp., analysed in this study. In fact, leaf reduction is proposed as one the most relevant traits to withstand water deficit (Peguero-Pina *et al.*, 2014). On the other hand, the feature of sclerophyllous leaves is considered as a functional adaptation to tolerate water stress under drought conditions (Savé *et al.*, 1999; Sardans *et al.*, 2013). This feature is not present

either *Q. pyrenaica* or *Q. faginea* or *Q. robur*, which could also justify a higher survival in *Q. ilex* and *Q. suber* under drought conditions.

Regarding *Q. ilex* interpopulation variability, eastern populations (Ja and Gr) showed a lower damage and mortality than western ones (Ca, Co and Se) (Figure 1), in agreement with the results observed by Navarro-Cerrillo *et al.* (2018). At plant individual level, not all the *Q. ilex* seedlings from the same population had a homogeneous behaviour, and both live and dead individuals in different percentages were observed at the end of the experiment (around 70% in Ca, Co, and Se, and 50% in Gr, Ja). These data show the existence of neighbouring individuals with different phenotypes in terms of abiotic stress tolerance. Thus, the percentage of live and dead individuals differing between populations should be used as an indication of the response to this abiotic stress in this species. The use of this indicator together with the quality of microhabitats would increase the survival of *Q. ilex* seedlings in the field. Seedlings located under the canopy of oaks, pines or adult pines in afforestation stands showed higher seedling growth than tall shrub and open sites, indicating that sheltered microhabitats are more suitable for oak establishment (Gómez, 2004).

4.2. Leaf water status in droughted Q. ilex seedlings as a physiological parameter of differences in tolerance

RLWC as a parameter of plant water status, has been related to drought tolerance in *Quercus* (Peguero-Piña *et al.*, 2009). We did not observe

differences in irrigated seedlings among populations. Water withholding caused a decrease in RLWC determined at day 25 to 43.1 – 70.1% depending on the population. Gr and Ca populations showed the highest and lowest RLWC, which in agreement with Valero-Galván *et al.* (2013). However, our results differ from those by Villar-Salvador *et al.* (2004) who found no changes in RLWC in non-irrigated *Q. ilex*. As stated above, the climate conditions beyond the watering regime will also determine the effect and the response to drought. In any case, it has been reported that *Q. ilex* has a high capacity of maintaining leaf turgor under severe drought (Forner *et al.*, 2018).

4.3. Photosynthesis parameters in droughted Quercus seedlings and tolerance differences

The F_v/F_m parameter reflects the amount of light energy required for photosynthesis that is used as a parameter of response to drought stress and tolerance as different studies have revealed (Peguero-Piña *et al.*, 2009; Sancho-Knapik *et al.*, 2018). As expected, irrigated seedlings showed optimal values for different plant species of around 0.83 (Maxwell and Johnson, 2000). Under water deficit, a decrease in photosynthesis, and hence demand in ATP and NADPH, occurs, which is accompanied by a decrease in electron flux from PSII to the quinone acceptor, thus reducing F_v/F_m value (Baker and Rosenqvist, 2004). This is the pattern observed in the present work and reported in previous publications for *Q. coccifera*, *Q. pubescents*, and *Q. ilex* (Méthy *et al.*, 1996; Peguero-Piña *et al.*, 2009; Vilagrosa *et al.*, 2010; Ramírez-Valiente *et al.*, 2019). Clear differences among *Quercus* spp.

were observed. Thus, *Q. faginea* and *Q. pyrenaica* seedlings showed F_v/F_m values near zero at, respectively, days 6 and 10, as was also observed above in the damage appearance and dead seedlings. As expected, dead oaks grouped in 5 scale did not show F_v/F_m values. At the end of the experiment, a 70 and 40% reduction in F_v/F_m took place in *Q. robur, Q. suber* and *Q. ilex,* respectively. F_v/F_m has been employed as a parameter of *Quercus* performance under drought conditions (Peguero-Piña *et al.,* 2009; Fernández-Marín *et al.,* 2017), although in some cases different results when comparing species, e.g. *Q. ilex* and *Q. suber,* have been reported (Ramírez-Valiente *et al.,* 2019). This can be due to either the severity of the stress, as well as the genotype, developmental stage, or the climate conditions employed. Thus, Méthy *et al.* (1996) determined that F_v/F_m was only affected when oak seedlings are subjected to an intense drought, with leaf predawn water potential < -4 MPa, a value that is rarely observed on mature trees, and Quero *et al.* (2006) have clearly shown that irradiance affects photosynthesis performance under drought conditions. By using the F_v/F_m parameter, we did not find statistically significant differences among *Q. ilex* populations. This can be interpreted as alive non-damaged seedlings, independently of its geographical origin showed similar responses to drought conditions, at least at the photosynthetic level.

Lack of water generally induces stomata closure, causing a decrease in the photosynthesis rate and stomatal conductance (Merilo *et al.,* 2014), factors related to drought tolerance (Martin *et al.,* 2019). The quantitative response depends on the species analysed (Acherar and Ramblal, 1992). In the present

study, at day 9, *A* and *Gs* decreased in the non-irrigated seedlings for all the *Quercus* spp., except in *Q. ilex,* where *A* values did not show significant differences between irrigated and non-irrigated individuals. *Quercus ilex* recorded higher values of *A* and *Gs* than the rest of the species analysed in this study, indicating that *Q. ilex* delays the stomatal closure under drought conditions (Vaz *et al.*, 2010; Barbeta *et al.*, 2016), correlating with the highest level of tolerance for this species observed in visual damages, dead seedlings and F_v/F_m values.

Net photosynthesis rate and stomatal conductance were reduced under drought conditions at different degrees on the surveyed *Q. ilex* populations, with statistically significant differences among them. At day 21, Ca showed the highest *A* and *Gs*, and Se the lowest *A* one (Figure 5E and F). The other populations had intermediate values for both parameters. The different behaviour previously discussed of eastern and western populations was not observed while using these two parameters. The Ca population, where most dead plants were observed, correlated with the lowest leaf water content and the highest stomatal conductance, thus suggesting it to be the least tolerant population amongst the studied ones.

4.4. Leaf chemical composition in Seville Q. ilex seedlings

Under drought conditions, all the compounds analysed remained in the range of those corresponding to irrigated seedlings (Figure 6), indicating a correct function of the metabolism and metabolic homeostasis. There were no statistically significant differences in the content of photosynthetic pigments,

chlorophylls, and carotenoids between treatments, suggesting little or no damage to the photosynthesis apparatus (Epron and Dayer, 1992; Gallé *et al.,* 2007). This observation could be species, genotype and/or experiment dependent, as in a previous study, a decrease in chlorophyll and an increase in carotenoids was reported for three *Quercus* spp., *robur, coccifera,* and *ilex* (Spyropoulos and Mavrommatis, 1978).

An increase in the content of sugar, amino acids and total phenolics was observed, which is considered an expected effect in those species prone to drought (Rivas-Ubach *et al.,* 2014). These compounds contribute to plant responses to biotic and abiotic stresses and to the survival of the seedlings, preventing water loss and enhancing osmoprotection under drought conditions (Rivas-Ubach *et al.,* 2014). The increase in the sugars, which act as osmolytes, could be explained by the mobilization of seed starch (Rodríguez-Calcerrada *et al.,* 2018; Simova-Stoilova *et al.,* 2018). Previous studies showed that starch reserves were depleted in the conversion to soluble sugars during drought (Simova-Stoilova *et al.,* 2018). Amino acids, such as proline and glycine, have been described as active osmotic compounds, and their increase in response to drought has been previously reported in *Q. ilex* (Rodríguez-Calcerrada *et al.,* 2018).

Several phenolic compounds with an antioxidant function have been described in *Q. ilex*, such as gallic acid, isoloquiritigenin or catechin (López-Hidalgo *et al.,* 2018). Our study found a significant increase in the content of phenolics in the non-irrigated seedlings, as previously reported (Rivas-Ubach *et al.,* 2014). This induction could be directly related to a direct

response to scavenge the increase in the levels of reactive oxygen species caused by drought. However, anthocyanins displayed a significant decrease under drought conditions that was contrary to previous published results (Spyropoulos and Mavrommatis, 1978), although a direct relationship between anthocyanin accumulation and drought tolerance does not always happen (Hughes *et al.*, 2010).

4.5. Management Implications

These results highlight the relevance of identifying drought tolerant seedlings for reforestation purposes in the Mediterranean area. The eastern Andalusian *Q. ilex* populations would be considered as better candidates for the selection of plant material as they were more tolerant to drought than the western ones. By using the described methodology, a quick screening for tolerance with a high number of individuals can be done at the nursery 6-month after acorn harvesting (June-July, when temperature is high) and a few months before transplanting to the field (October-November). At the same time, a hardening effect can be possible. In addition, it is used in our research group for analysing multi-stress responses (e.g. drought and *Phytophthora*), and collecting plant tissue (root and leaf) for molecular studies in order to identify key genes and gene products implicated in the response.

5. Conclusions

Summer environmental conditions reached in Southern Spain (water withholding, high temperature, solar irradiance, and low humidity) together

with the novel use of perlite as the growth substrate), allowed the imposition of a rapid and severe drought stress, which enabled us to establish the differences in drought tolerance among *Quercus* spp. and the variability among *Q. ilex* populations. Out of the species evaluated, *Q. ilex* behaved as the most tolerant species to drought, as deduced from the analysis of its visual symptoms, leaf water status, photosynthesis parameters, and leaf chemical composition. The *Q. ilex* seedlings that survived under intense drought stress for 28 days adjusted its physiology and metabolism to cope with arid conditions. These seedlings responded with a delay in stomata closure, leaves well hydrated and less pronounced damages in the photosynthetic apparatus that allowed to be photosynthetically active. Even under severe drought stress, the photosynthetic machinery was not altered, as demonstrated by no significant changes in the concentration of photosynthetic pigments. Also, an increase in the levels of sugars and amino acids, were observed, supporting active metabolism and metabolite homeostasis. Differences in tolerance among *Q. ilex* populations were deduced from visual estimation of damage and seedling mortality, with the eastern being more tolerant than the western ones. This difference was not clearly manifested at the leaf water status and photosynthesis parameters, thus indicating that living, non-damaged seedlings showed a similar pattern, independently of their geographical origin. So, either inter-, or intrapopulation variability in drought tolerance do exist, with differences among populations determined by the percentage of tolerant individuals. This present study suggests several indicators (damage symptoms, mortality rate, leaf water content, photosynthetic, and biochemical parameters) that

can be used in the selection of resilient genotypes in breeding and reforestation programs, especially under climate change conditions. The probability of a seedling to survive under drought conditions will depend largely on the intraspecies genetic variability. Thus, in a reforestation program, a previous analysis of survival in 6-month-old seedlings under drought conditions will allow to know those individuals that will tolerate the first summer drought since *Quercus* species are very vulnerable to stressed conditions during their early stages of life.

6. Supplementary Materials

Table S1: Statistical values obtained in leaf photosynthesis parameters in *Quercus* spp. (A) and Andalusian *Q. ilex* populations (B) at day 9, and Andalusian *Q. ilex* population (C) at day 21. Asterisk indicates that the Kruskall-Wallis´s test was carried out obtaining *F* values.

A	***A***		***Gs***	
	T	***p-value***	***T***	***p-value***
Q. faginea	5.65	0.0048	4.20	0.0137
Q. pyrenaica	6.39	0.0031	28.99	0.0000
Q. robur	8.97	0.0009	12.73	0.0002
Q. suber	11.83	0.0003	5.54	0.0052
Q. ilex	1.69	0.1672	3.78	0.0194
B	***A***		***Gs***	
	T	***p-value***	***T***	***p-value***
Ca	-0.04	0.9732	1.22	0.2879
Co	3.03	0.0387	4.28	0.0129
Se	-0.99	0.3790	1.11	0.3295
Gr	0.27	0.8020	8.49	0.0011
Ja	3.29	0.0303	6.36	0.0031
C	***A***		***Gs***	
	T*	***p-value***	***T****	***p-value***
Ca	5.23	0.0064	7.83	0.0014
Co	13.5*	0.0213	27.00*	0.0065
Se	13.5*	0.0213	22.00	0.0000
Gr	9.94	0.0006	18.00*	0.0132
Ja	13.5*	0.0213	36.00*	0.0039

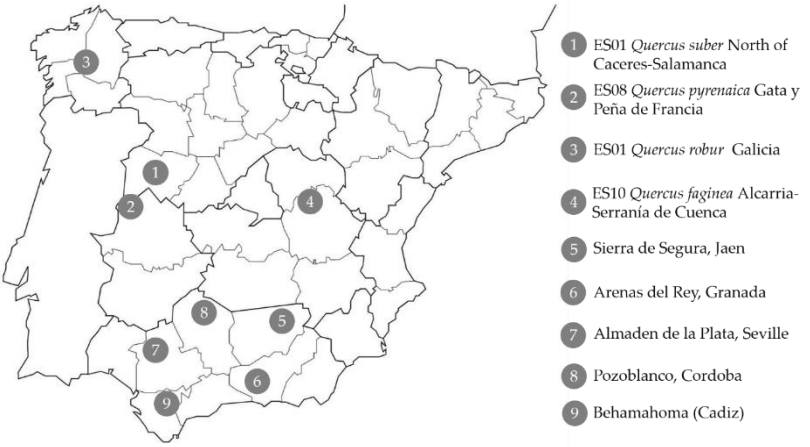

Figure S1: Location of all *Quercus* spp., and Andalusian *Q. ilex* population used in this study.

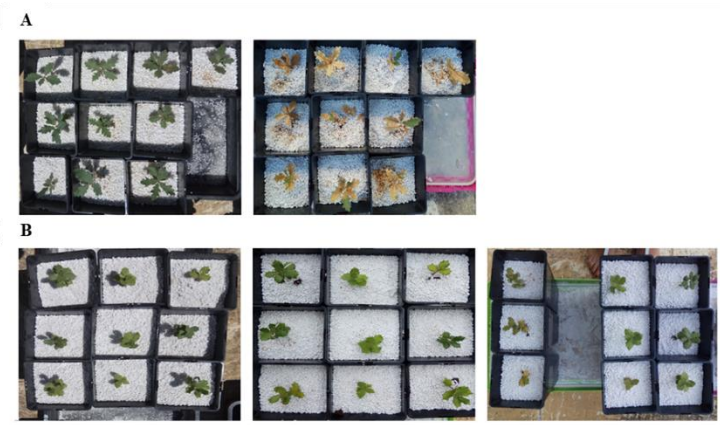

Figure S2. Visual evaluation of damage symptoms in the two most contrasting species: *Q. pyrenaica* **(A)** and *Q. ilex* **(B)**. Pictures were taken at days 1 and 9 in *Q. pyrenaica* and at days 1, 9 and 27 in *Q. ilex*; whereas all the seedlings for *Q. pyrenaica* were dead, no clear damage symptoms were observed in any of the *Q. ilex* seedlings.

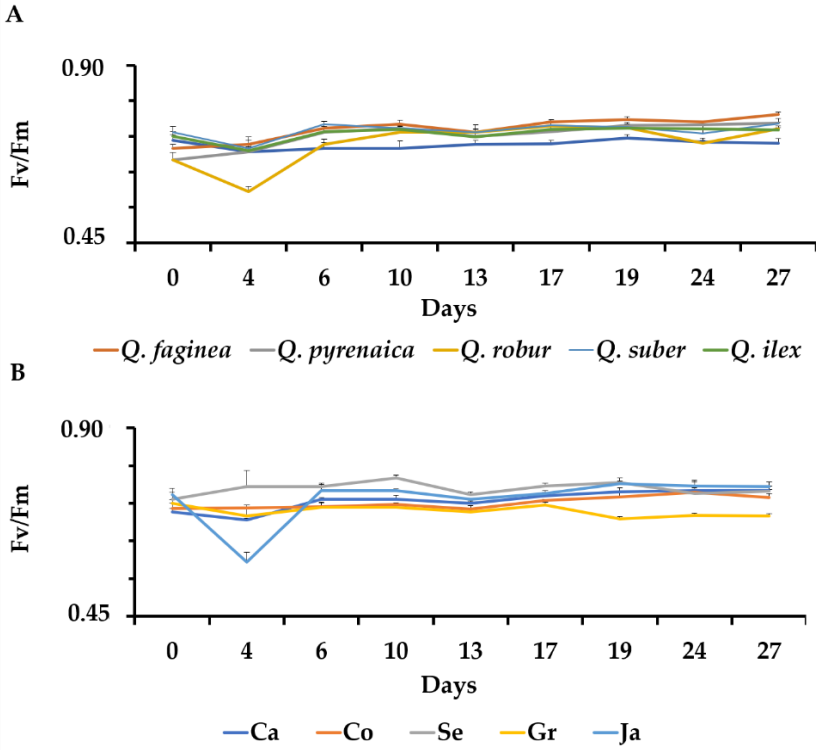

Figure S3. Measurements of quantum yield of photosystem II (F_v/F_m) in dark adapted leaves from *Quercus* spp. **(A)**, and *Q. ilex* interpopulation species **(B)** (irrigated seedlings) during drought progression. Values are mean ± SE of three biological replicate.

Chapter III

Proteomics data analysis for the identification of proteins and derived proteotypic peptides of potential use as putative drought tolerance markers for *Quercus ilex*

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Abstract

Drought is one of the main causes of mortality in holm oak (*Quercus ilex*) seedlings used in reforestation programs. Although this species shows high adaptability to the extreme climate conditions prevailing in Southern Spain, its intrinsic genetic variability may play a role in the differential response of some populations and individuals. The aim of this work was to identify proteins and derived proteotypic peptides potentially useful as putative markers for drought tolerance in holm oak by using a targeted post-acquisition proteomics approach. For this purpose, we used a set of proteins identified by shotgun (LC-MSMS) analysis in a drought experiment on *Q. ilex* seedlings from four different provenances (viz. the Andalusian provinces Granada, Huelva, Cadiz and Seville). A double strategy involving quantification of proteins and target peptides by shotgun analysis and post-acquisition data analysis based on proteotypic peptides was used. To this end, an initial list of proteotypic peptides from proteins highly represented under drought conditions was compiled that was used in combination with the raw files from the shotgun experiment to quantify the relative abundance of the fragment's ion peaks with the software Skyline. The most abundant peptides under drought conditions in at least in two populations were selected as putative markers of drought tolerance. A total of 30 proteins and 46 derived peptides belonging to the redox, stress related, synthesis, folding and degradation, and primary and secondary metabolism functional groups were thus identified. Two proteins (viz., subtilisin and chaperone GrpE protein) were found at increased levels in three populations, which make them

especially interesting for validation drought tolerance markers in subsequent experiments.

Keywords: peptide markers; *Quercus ilex*; drought tolerance; targeted post-acquisition proteomics.

1. Introduction

Holm oak (*Quercus ilex*) is the dominant tree species in natural forest ecosystems over the Western Mediterranean Basin, as well as in the agrosilvopastoral Spanish "*dehesa*", which is environmentally, economically and socially important (Olea and San Miguel-Ayanz, 2006; Abril *et al.*, 2011). This species is highly adaptable to drought, and to the high temperatures and irradiation typical of Southern Spain. However, the main cause of mortality in holm oak plantations is water deficiency, with drought stress acting as a major factor of decline (Crescente *et al.*, 2002; Echevarria-Zomeno *et al.*, 2009). This situation can be expected to worsen in a scenario of climate change where statistical models have predicted that 40% of the land areas with a high density of *Q. ilex* will be unsuitable for its survival (Keenan *et al.*, 2011).

Studies on the genetic variability associated with both environmental factors and genotypes have shown high population variability and polymorphism in *Quercus* spp. (Castro-Diez *et al.*, 1997; Lumaret *et al.*, 2009; Valero-Galván *et al.*, 2010; Valero Galvan *et al.*, 2011). Also, *Q. ilex* has been shown to exhibit high variability in traits associated with drought tolerance, both within and between populations (Ramirez-Valiente *et al.*, 2009). Because this is a non-domesticated species with a very long-life cycle, it is not amenable to conventional plant breeding. Therefore, for management and conservation practices based on resilient, elite, genotypes of holm oak trees to be effective, they must rely on a sound knowledge of their biology and molecular mechanisms of adaptation to adverse climatic conditions. The

response of plants to stress related situations may, in theory, be improved by characterizing their biodiversity and the selecting elite genotypes based on specific molecular markers. This approach can be quite challenging with orphan species such as holm oak, which has a still incompletely sequenced genome and largely unexplored molecular features (Jorge *et al.,* 2006; Valero-Galvan *et al.,* 2013; Jorrín-Novo *et al.,* 2014; Rico *et al.,* 2014; Rivas-Ubach *et al.,* 2016; Guerrero-Sanchez *et al.,* 2017; Fernández i Marti *et al.,* 2018; Lopez-Hidalgo *et al.,* 2018; Natali *et al.,* 2018; Guerrero-Sanchez *et al.,* 2019; Romero-Rodriguez *et al.,* 2019). Fortunately, omics approaches have enabled crucial advances in these directions. Thus, some multiomics studies have addressed *Q. ilex* (Lopez-Hidalgo *et al.,* 2018); also, a reference transcriptome for this species has been generated (Guerrero-Sanchez *et al.,* 2017; Guerrero-Sanchez *et al.,* 2019) and, more recently, the metabolome of acorn determined (Lopez-Hidalgo *et al.,* 2021). In any case, the greatest efforts have focussed on the proteomics of *Q. ilex*. A number of the proteomic studies have used 1D and 2D gel-based analysis to investigate drought tolerance in this species (Jorge *et al.,* 2006; Valero-Galvan *et al.,* 2013; Simova-Stoilova *et al.,* 2015). Also, recent studies have addressed various aspects of its biology by using shotgun (LC-MS/MS) proteomic analysis (Lopez-Hidalgo *et al.,* 2018; Romero-Rodriguez *et al.,* 2019; Gomez-Galvez *et al.,* 2020). In addition, species-specific improved databases such as the recently compiled holm oak transcriptome database (Guerrero-Sanchez *et al.,* 2017; Guerrero-Sanchez *et al.,* 2019), and other sequenced *Quercus* species databases such as those for *Q. robur* (Plomion *et*

al., 2016) and *Q. suber* (Ramos *et al.,* 2018), have substantially expanded available knowledge of holm oak biology.

Quantitative proteomics is providing increasingly powerful tools for identifying markers of complex traits. Thus, identifying target peptide signals against mass spectrometry libraries is an efficient method for protein identification and quantification (Gillet *et al.,* 2012). Targeted proteomics, however, does not allow identification of new proteins as it requires the prior measurement of the targeted proteins by discovery proteomics; rather, it is useful for detecting changes in the protein abundances from previously acquired information (Domon and Aebersold, 2010). Therefore, this proteomics branch can be useful to characterize coordinated changes in protein abundance with a view to identifying or validating proteins as markers for specific traits. Recently, proteotypic peptides have proved useful for protein quantification (Escandon *et al.,* 2021).

In this work, we used a double strategy for proteins and peptides quantification by targeted post-acquisition data analysis against a species-specific *Q. ilex* database with a view to identifying proteotypic peptides of potential use as putative drought tolerance markers for holm oak. For this purpose, a set of raw data generated in a shotgun experiment performed after 17 and 24 days under drought condition in *Q. ilex* seedlings from four different provinces in Andalusia was used. Based on previous studies of interpopulation variability of this species (Valero-Galvan *et al.,* 2013; Fernández i Marti *et al.,* 2018; Navarro-Cerrillo *et al.,* 2018; San-Eufrasio *et*

al., 2020) we selected two populations from the southeast (Cadiz, Granada) and two from the northwest (Huelva, Seville) of Andalusia, with the purpose of identifying changes in proteins and derived peptides persistent over time in response to drought in different populations. In this work, various proteins and peptides are proposed as putative markers of drought tolerance in holm oak that transcend not only the tolerant phenotype but also populations and examined in biological terms.

2. Materials and Methods

2.1. Proteomic dataset

The dataset used was compiled from the leaf proteome of *Q. ilex* seedlings in four different Andalusian populations, Southern Spain, namely: Granada (G), Huelva (H), Cadiz (C) and Seville (S). A detailed map of the Andalusian locations from which samples were collected is shown in Figure S1. All populations were under severe drought stress conditions, such as those imposed in summer under Mediterranean climate (Table S1). Detailed information about specimen provenances (Table S1), plant growth, stress conditions imposed and physiological measurements can be found elsewhere (San-Eufrasio *et al.,* 2020). Briefly, acorns were germinated and grown under greenhouse conditions in perlite according to Simova-Stoilova *et al.* (2015). Severe drought was imposed by withholding water for 28 days at the 10-leaf stage under the following experimental conditions: 46/22 °C, 28 MJm^{-2}/day, 41% HR (San-Eufrasio *et al.,* 2020). Two different sampling times based on measured leaf physiological parameters were used for proteomic analysis. Thus, leaves were collected after 17 and 24 days of

drought, corresponding to an average drop in chlorophyll a fluorescence of 20% and 40%, respectively, in droughted seedlings relative to well-watered seedlings in all populations (San-Eufrasio *et al.*, 2020) (Figure S2). These sampling times were selected as representative of an early and later stage of the response to drought with photosynthetically active leaves. Figure S3 shows visual damage symptoms observed in the seedlings 25 days after drought. Healthy leaves from different seedlings under different conditions as regards population, treatment and sampling time were collected, frozen in liquid nitrogen and stored at −80°C for subsequent proteomic analysis.

2.2. Protein extraction and mass spectrometry analysis

Five fully expanded (photosynthetically active) leaves per plant from each population (G, H, C, S), treatment (control well-watered, Control and drought) and sampling time (17 and 24 days) were crushed with liquid nitrogen and used for protein extraction. Proteins from three independent biological replicates (200 mg of fresh tissue each) were extracted with TCA/acetone-phenol (Wang *et al.*, 2006), solubilized in a solution containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% (w/v) Triton X-100 and 100 mM DTT, and quantified by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard.

Shotgun analysis was performed by using 90 µg of BSA protein equivalents per sample that were prefractionated in SDS-PAGE according to Valledor and Weckwerth (2014). The resulting unique bands were excised from the gels and digested with proteomics-grade trypsin (Promega) to a final

concentration of 12.5 ng/µL according to Romero-Rodríguez *et al.* (2019). Digested peptides were desalted by passage through C18 cartridges from Scharlau (Barcelona, Spain) and eluted peptides were vacuum dried and dissolved in a mixture of 70:30 (v/v) acetonitrile (ACN)/water containing 0.1% trifluoroacetic acid. Mass spectrometry analyses were conducted at the Proteomics Facility for Research Support Central Service (SCAI) of the University of Cordoba (Spain), using a Dionex Ultimate 3000 nano UPLC instrument from Thermo Scientific (San Jose, CA, USA) coupled to a nanoelectrospray ionization source and a trihybrid analyzer Thermo Orbitrap Fusion mass spectrometer, also from Thermo Scientific, operating in the positive ion mode. The specific settings used in the LC-MS/MS analyses are described elsewhere (Meyer and Schilling, 2017).

2.3. Protein identification and quantification

The raw data obtained from the MS analysis were processed with the software Proteome Discoverer v. 2.1.0.81 from Thermo Scientific. MS2 spectra were searched with the SEQUEST engine against the FASTA database obtained from the *Q. ilex* transcriptome (Guerrero-Sanchez *et al.*, 2017; 2019). Precursor mass tolerance was set at 10 ppm and fragment ion mass tolerance fixed at 0.1 Da. Only those ions with a charge state of +2 or greater were used. *In silico* peptide lists were generated by theoretical tryptic digestion, allowing up to two missed cleavages, carbamidomethylation of cysteines as a fixed modification and oxidation of methionine as a variable modification. Peptides were classified into proteins groups according to the law of parsimony and filtered to FDR = 5% and XCorr ≥ 2.

Proteins were quantified in relative terms from the peak areas for precursor ions (specifically, the average of the three strongest peptide ion signals) from the identified peptides were used (Al Shweiki *et al.*, 2017). Protein values were then normalized by using a method that accounts for variability between runs to normalize relative protein abundance between samples, using the sum of the peak area values for each sample. Only those values consistently present in the three biological replicates were considered for further statistical analysis. Proteins were categorized by function by using the protein FASTA sequences in the software MERCATOR (https://www.plabipd.de/portal/mercator4) (Lohse *et al.*, 2014), an online tool for batch classification of proteins or gene sequences into Map-Man functional plant categories. In addition, nonannotated proteins were subjected to GO enrichment by using the Panther tool (http://pantherdb.org/).

The raw mass spectrometry data thus obtained were deposited on the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Perez-Riverol *et al.*, 2019) with the dataset identifier PXD023782.

2.4. Statistical analysis of data and selection of target proteins

A Partial Least Squares-Discriminant analysis (PLS-DA) of the entire dataset was performed to explain variance and correlation between the different variables, using the software RStudio v. 4.0.3 (Caret package v. 6.0-76) (Kuhn, 2020). Target proteins were selected and *in silico* data were analyzed by using the entire dataset provided by the shotgun analysis. All proteins selected met the following criteria: (*a*) the entire dataset was filtered by confidence parameters (score ≥ 2, at least 2 peptides per protein); and (*b*) they were the best represented in qualitative and/or quantitative terms ($p \leq 0.05$) under drought conditions at both sampling times in at least two populations. Figure 1 illustrates the experimental workflow.

2.5. Targeted post-acquisition data analysis for selection of putative peptides markers

A list of target peptides generated from the proteotypic peptides (specific peptide sequences from the selected proteins as far as existing annotation information allowed for) was compiled. Proteotypic peptides were searched by aligning the sequences on the entire *Q. ilex* database, using the Bash (Bourne-again shell) tool (http://ftp.gnu.org/gnu/bash/) (Altschu *et al.*, 1990) and the Blast protein (blasp) tool for Ubuntu (https://www.exoscale.com/syslog/blast/). Proteotypic peptides were quantified by integrating the areas of the chromatographic fragment ion peaks in Skyline software (https://skyline.ms). The parameters used for relative quantification of MS1 were 0.055 *m/z* mass tolerance for instrument, 0.5 *m/z* for library peak integration and a resolution of 120 000 at *m/z* 200.

The integration peak and retention time (RT) for each peptide were checked by hand in order to confirm the reproducibility of ions among samples. Peptide values were subsequently assessed for statistical significance by using the external tool MS Stats in Skyline (Chang *et al.*, 2012). Data were normalized by equalizing intensity medians and then subjected to log2-transformation, after which ANOVA analysis was used to select the best represented proteins among those exhibiting significant changes ($p \leq 0.05$) under drought conditions. Figure S4 provides a quantitative depiction of the proteins and precursor ions.

A protein interaction network of the selected proteins was generated by using the web tool STRING10 (http://string-db.org). The protein homologs in *Arabidopsis* were analyzed by sequence BLASTing of the TAIR database (http://www.arabidopsis.org/Blast/index.jsp), followed by application of STRING10 to develop a proteome-scale interaction network (Suo *et al.*, 2015).

3. Results

3.1. Qualitative and quantitative analysis of drought stress responsive proteins

Shotgun analysis allowed a total of 4470 proteins to be identified in the *Q. ilex* leaf proteome (Table S2; data are available via ProteomeXchange with identifier PXD023782) of which 2920 fulfilled the following criterion for confident identification: XCorr ≥ 2 and at least two different peptides per protein. An overall 2261 proteins were deemed variable in accordance with

the following confidence criteria: *a*) consistent presence in all replicates, *b*) statistical significance (FDR < 0.05) and *c*) drought/control ratio ≥ 2 and ≤ 0.5. In this group, 1692 proteins exhibited qualitative changes in at least one population and sampling time, and 569 quantitative changes, 1683 being more abundant in droughted seedlings. The initial dataset was screened to select those variable proteins most markedly represented under drought conditions at both sampling times in each population. A total of 380 proteins were thus screened, of which 48 were present in at least two populations and deemed markers candidates. A schematic view of the workflow as well as the details of the experimental design are shown in Figure 1 and Figure S5.

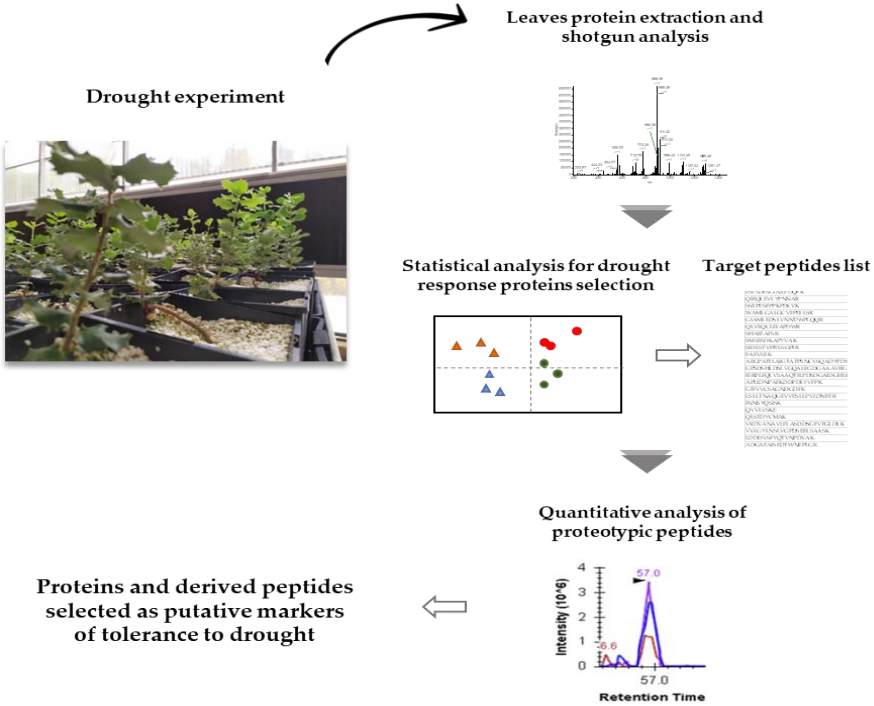


Figure 1. Schematic workflow for selection of putative markers of drought tolerance.

Multivariate analysis integrating the entire dataset is shown in Figure S6A, where the first two components (25% of the total variability) separated populations. A hierarchical clustering was also performed and represented in a dendrogram (Figure S6B), in which two main clusters were observed: one grouped Huelva and Granada populations, and the other grouped Seville and Cadiz, independently of the sampling time. Replicates of the same experimental condition (population, treatment and sampling time) were grouped, demonstrating the reproducibility throughout the experiment. To check the effect of stress on the populations, the sampling times were analyzed separately by partial least squares regression analysis (PLS-DA) (Figure 2). The first two components explained 20% (first sampling time) and 31% (second sampling time) of the total variability. Component 1 resolved Cadiz control plants, and component 2 resolved plants under drought for 17 days (first sampling) from control plants except in the Huelva population (Figure 2A). For the second sampling time (24 days) component 1 resolved the population pairs Huelva–Granada and Seville–Cadiz, and component 2 resolved treatments (Figure 2B). Based on this analysis, a clearer effect of drought treatment and populations can be appreciated at the second sampling time.

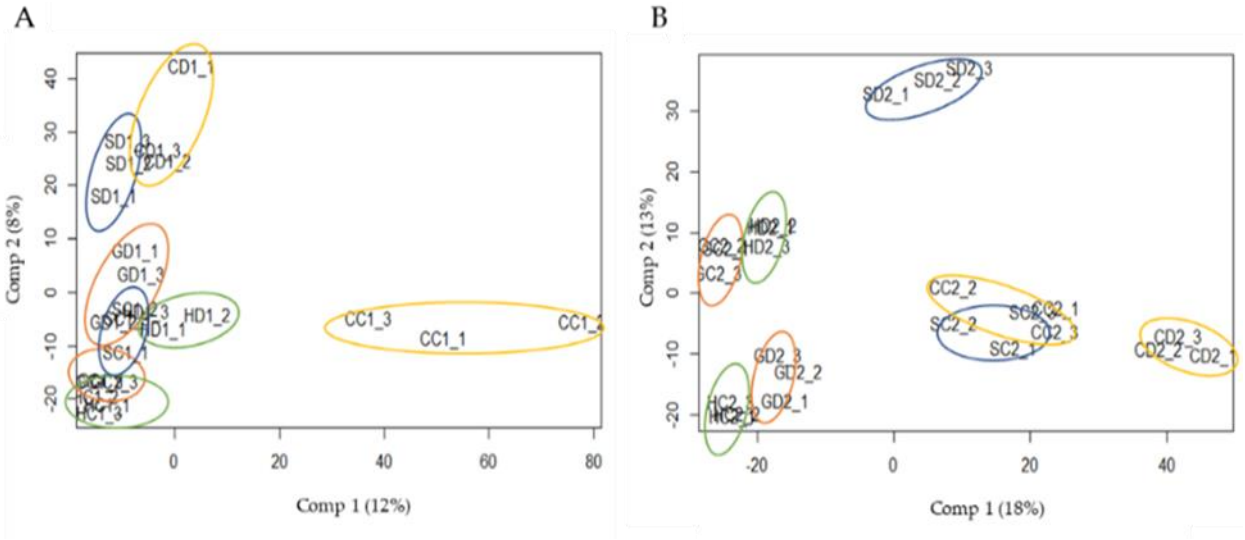


Figure 2. Partial least squares discriminant analysis (PLS) of the entire dataset after 17 **(A)** and 24 days of drought **(B)** is shown in the upper part. Cumulative proportion of variance explained by components is shown below. C, Cadiz; G, Granada; H, Huelva; S, Seville. The letters following C, G, H, or S denotes treatment (D, drought; C, control), the numbers before the underscore sampling time (1, 17 days; 2, 24 days) and that after it replicates (1, 2 or 3)

Figure 3A shows the 2261 variable proteins significantly (FDR < 0.05) up- or down-accumulated (twofold change) in the drought group and Figure 3B a Venn diagram of the 380 variable proteins among them. The Granada and Huelva populations exhibited the greatest number of unique variable proteins (107 each), followed by Seville (71) and Cadiz (47). Huelva and Seville shared the largest number of variable proteins (16), followed by Granada-Huelva (14), Huelva-Cadiz (8), Seville-Cadiz (8), Granada-Seville (4) and Granada-Cadiz (2). Only two proteins changed significantly by effect of drought in three populations; thus, GrpE protein (qilexprot_13677) changed in Huelva, Seville and Cadiz, and subtilisin-like protease (qilexprot_25223) in Granada, Seville and Cadiz.

The previous 380 variable proteins were characterized in functional terms by using Mercator (Lohse *et al.*, 2014) and GO enrichment (http://pantherdb.org/) for classification into 16 main groups (Figure 4A), namely: energy, carbohydrate, amino acid, lipid, hormone, coenzyme and secondary metabolism, other metabolic processes, cellular processes, folding-sorting and degradation, synthesis (transcription/translation), structural, defense and response to stress, redox, signalling and transport. The best represented functional group was synthesis (62), followed by folding-sorting and degradation (42), defense and response to stress (37), carbohydrate metabolism (33) and redox (29), the previous five groups accounting for more than 50% of all identified proteins. Figure 4B compares

the proteins whose abundance was altered by drought in each population as grouped by functional category.

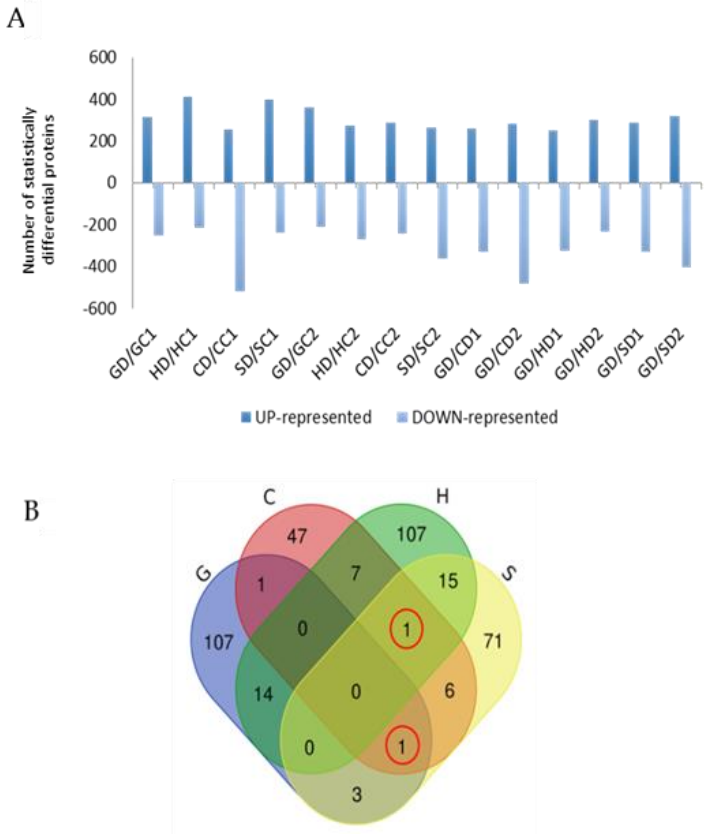


Figure 3. Variable proteins significantly (FDR < 0.05) up- or down-accumulated (twofold change) under drought conditions (**A**); C, Cadiz; G, Granada; H, Huelva; S, Seville. The letter following C, G, H or S denotes treatment (D, drought; C, control) and the number sampling time (1, 17 days; 2, 24 days). Venn diagram showing significantly up-represented proteins under drought conditions in each population (**B**).

3.2. Targeted data analysis for selection of peptides as putative markers of drought tolerance

A list of proteotypic peptides derived from the 48 selected proteins was compiled for subsequent targeted analysis. The list, which included 219 proteotypic peptides with a charge state of +2 or higher was used for quantification with the software Skyline as described in Section 2.5. As confirmed by Figure S7, using the above-described library allowed 159 peptides to be successfully integrated with a robust, reproducible, high-quality peak shape in all three replicates (Table S3). A statistical analysis of variance (ANOVA) on normalized data revealed 71 peptides comprising 32 different proteins, were significantly better represented in droughted seedlings. Those peptides and proteins better represented in at least two populations (46 peptides from 30 proteins) were selected as putative markers of drought tolerance (Table 1). Figure S4 illustrates graphically protein and peptide quantification. The most representative protein functions in the marker panel (Table 1) were synthesis and mRNA processing with 9 proteins. Seven proteins belonged to the redox and response to stress functional groups; 3 to the folding, sorting and degradation group; and 2 to the transport group. Other metabolic functional groups such as carbohydrate metabolism (2), secondary metabolism (1), photosynthesis (1) and other processes (5) were also represented. The Huelva population was that exhibiting the greatest number of proteins, most of which belonged to the synthesis and mRNA processing group (7), and the stress-related and secondary metabolism group (6). Other proteins associated with energy and

metabolism (viz., photosynthesis, carbohydrate metabolism and other cellular processes) were also represented (5). The second population as regards proteins changes was Seville, with proteins of the metabolism (6), synthesis (4), folding and degradation (3), and stress-related (3) groups. The Granada population exhibited smaller numbers of changing proteins, and only in the synthesis (5) and metabolism (3) groups. Finally, the Cadiz population was that exhibiting the least changes and mainly in stress-related (3), metabolic processes (2) and folding and degradation proteins (2). A protein-protein interaction network among the 48 proteins previously selected was performed using the web-tool STRING10 (http://string-db.org) (Figure 5). Strong connection between proteins of synthesis and those of folding and degradation was observed.

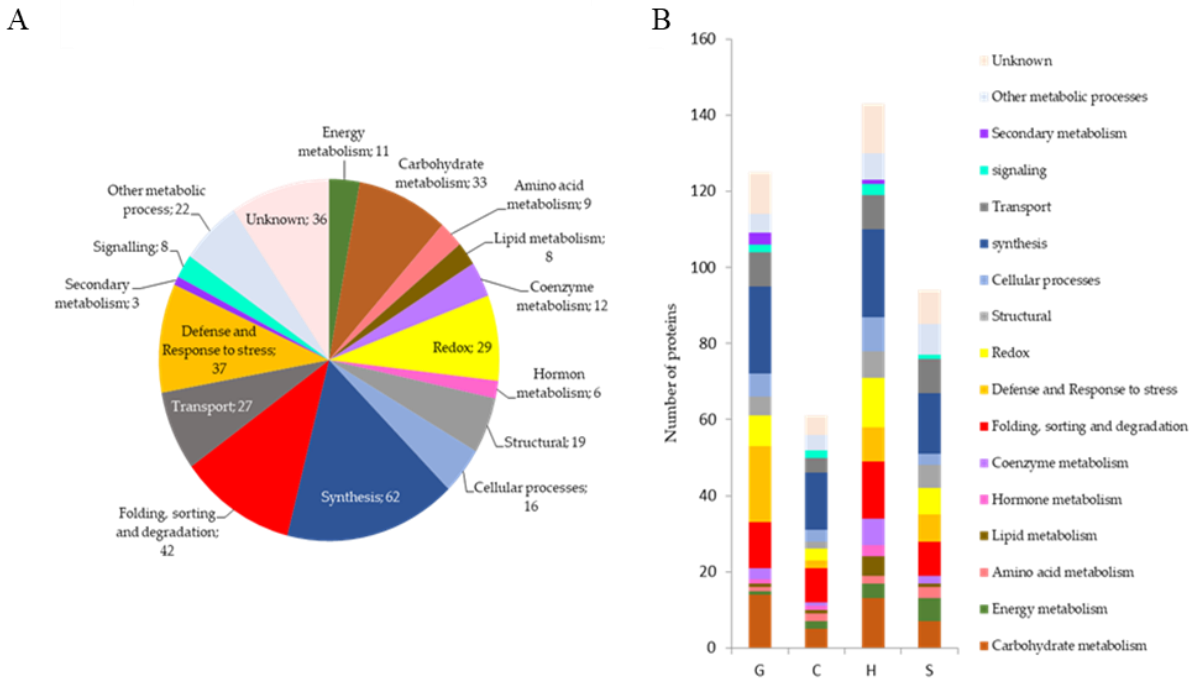


Figure 4. Functional categories of the 380 proteins. Total number of proteins significantly increasing in abundance after drought (**A**) and in each of the populations (**B**): C, Cadiz; G, Granada; H, Huelva; S, Seville.

Table 1. List of peptides and proteins selected as putative markers of tolerance to drought

Protein ID	Peptide sequences	Precursor *m/z*	Protein description	Protein function	Experimental condition showing significant change[a]			
qilexprot_45247	DAWDTSVLVEMK	697,333	Granule-bound starch synthase 1, chloroplastic /amyloplastic	Carbohydrate metabolism			S	G
	FSFSDFSLLNLPDQFK	952,971					S	G
	QIEQLEVLYPNNAR	843,940					S	G
qilexprot_71384	SSEPESFPPKPDLVK	828,924	Glycosyl hydrolase family protein with chitinase insertion domain	Carbohydrate metabolism		H	S	
qilexprot_18873	WAMLGALGCVFPELLSR	968,491	Chlorophyll a-b binding protein, chloroplastic	Photosynthesis		H	S	
qilexprot_32784	GIAMLEDSLVNNTSSPLQQR	1087,046	Mitochondrial fission 1 protein A	Cellular processes	C		S	
	QLVEQCLEIAPDWR	878,934			C		S	
qilexprot_49492	SHAIEAFSR	509,256	T-complex protein 1 subunit beta	Cellular processes			S	G
qilexprot_42362	SMSESDKAPYVAK	714,834	High mobility group B protein 4	Cellular processes		H	S	

qilexprot_14200	SIDLSTVHYLSGPIR	552,964	Formamidase	Other metabolic process	C	H		
qilexprot_39395	FAEVLEK	418,228	3-phosphoshikimate 1-carboxyvinyltransferase	Other metabolic process		H		G
qilexprot_29542	AEGPATILAIGTATPSNCVSQADYPDYYFR	1083,173	Chalcone synthase	Secondary metabolism		H		G
	GPSDSHLDSLVGQALFGDGAAAVIIGADPDTK	1031,844				H		G
	IERPLFQLVSAAQTILPDSDGAIDGHLR	1011,205				H		G
qilexprot_49771	APLIDNPAFKDDPDLYVFPK	1138,082	Calreticulin	Folding, sorting and degradation		H	S	
qilexprot_25223	GIFVVCSAGNDGDFK	793,366	Subtilisin-like protease	Folding, sorting and degradation	C		S	G
qilexprot_13677	LSLLTNAQGEVVESLLPVLDNFER	1328,700	GrpE protein	Folding, sorting and degradation	C	H	S	
	INNSYQSISK	577,300			C	H	S	
qilexprot_55000	QVVLVSKE	451,268	2-alkenal reductase (NADP(+)-dependent)	Redox	C	H		
qilexprot_1533	QLSTDYCMAK	616,764	Short-chain alcohol dehydrogenase A	Redox	C		S	
	VRDVANAVLFLASDDSGFVTGLDLK	874,459			C		S	
qilexprot_55764	VVLGYLNSLVGPDSEELSAASK	1124,585	Protein disulfide-isomerase	Redox		H	S	
	LDDDVSFYQTVNPDVAK	963,456				H	S	

	ADGAFAISEDTWNEPLGR	974,952	Endoplasmin homolog	Response to stress		H	S	
	EVTEEEYTK	564,255				H	S	
qilexprot_3345	FYHSLAK	433,228				H	S	
	YLNFLMGLVDSDTLPLNVSR	1142,084				H	S	
	IAEEDPDEANDKDK	794,849				H	S	
qilexprot_72159	NKDEHETTTTTTPGGNEGAVESK	801,364	Dehydrin	Response to stress		H		G
	EEEMASEFEK	614,752				H		G
qilexprot_48029	ELAENLFPEDDTVSQTPTLQSSENVLVR	1044,179	Senescence/dehydration-associated protein AT3g51250	Response to stress	C		S	
qilexprot_8871	KGCTPSQLALAWVHHQGK	673,013	Probable aldo-keto reductase 1	Response to stress		H	S	
qilexprot_19464	VNWAYASGQR	576,300	Oligouridylate-binding protein	mRNA processing	C			G
	SVVELTNGSSEDGK	711,300			C			G
qilexprot_70616	LITVTASENPDSR	701,900	BnaC03g49780D protein	mRNA processing		H		G
qilexprot_26698	DKPESDGADLANK	680,319	Zinc finger protein VAR3, chloroplastic	mRNA processing		H	S	
	SVASNAIEWTGNASGSSVPDK	524,745				H	S	
qilexprot_2527	IEDIDAYAPK	567,784	Myb domain containing transcription regulator	Synthesis	C		S	
qilexprot_7552	IVDVCEIGDSFIR	761,800	Proliferation-associated protein 2G4	Synthesis		H	S	
	ALQLVVSECKPK	686,383				H	S	

qilexprot_56656	ISFSGIDGKPEDVLNPK	605,650	Probable methionyl-tRNA synthetase	Synthesis	H		G
qilexprot_70881	VQDTYDTELAGK	670,319	Eukaryotic translation initiation factor 2c	Synthesis	H		G
qilexprot_71168	EDENRLDEVGYDDVGGVR	679,305	Transitional endoplasmic reticulum ATPase	Synthesis	H		G
qilexprot_68980	EFFGSENNSLVSAQVIFHENPR	840,737	RNA-binding family protein	Synthesis	H	S	
qilexprot_68567	SPPINEVVQSGVVPR	789,400	Importin subunit alpha	Transport	H	S	
qilexprot_4392	GLYENSGGGANVVNHGYTK	968,957	Aquaporin	Transport	H		G

[a] *Q. ilex* population in which significant changes occurred under drought stress: C, Cadiz; G, Granada; H, Huelva; S, Seville.

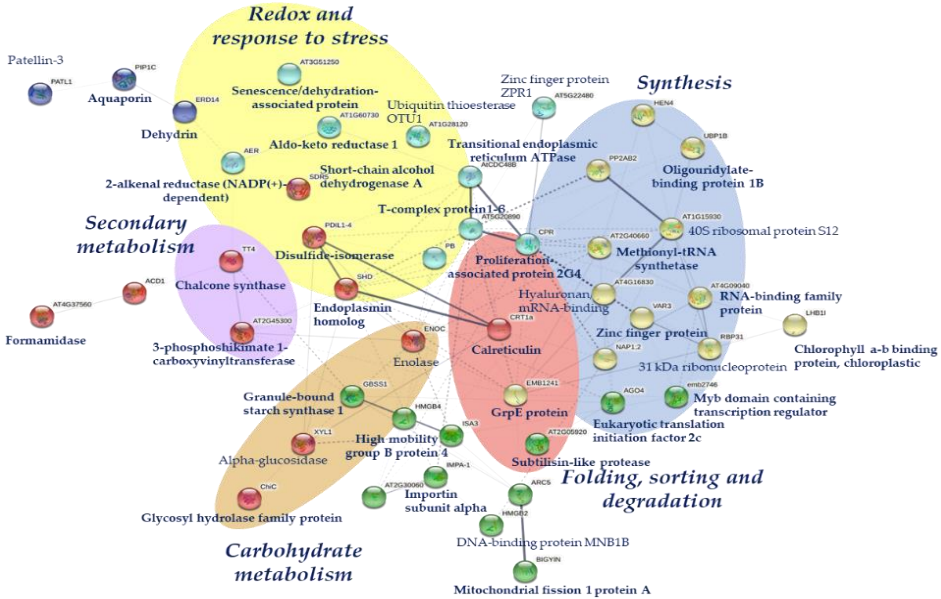


Figure 5. Analysis of the protein interaction network of the 48 proteins selected as putative markers of drought tolerance.

4. Discussion

In this work, we used a double proteomic strategy for protein and peptide quantification in order to identify putative protein markers associated with drought tolerance in *Q. ilex*. For this purpose, a dataset obtained by shotgun proteomics analysis of four *Q. ilex* populations from different provinces of Andalusia, Southern Spain, under severe drought stress (San-Eufrasio *et al.*, 2020) was analysed by using a double strategy, combining shotgun protein quantification of proteins and target peptides with post-acquisition analysis of data based on proteotypic peptides. The populations were selected based on previous studies of *Q. ilex* variability between eastern populations and western ones (Navarro-Cerrillo *et al.*, 2018; San-Eufrasio *et al.*, 2020), which demonstrated a relationship between tolerance and provenance. However, intrapopulation variability was also observed as corroborated by the study of morpho-physiological and biochemical parameters (San-Eufrasio *et al.*, 2020). For this reason, the aim of this work was the search for putative drought tolerance markers that transcend not only the tolerant phenotype but also populations.

The typically high complexity of proteomes makes protein identification by mass spectrometry irreproducible as a result of precursor ions being selected stochastically. Also, forest plants exhibit enormous biological variability that results in even poorer reproducibility among samples. Targeted proteomics analyses can be used to identify, characterize and quantify small sets of proteins previously selected by mass spectrometry analysis (Rodiger and Baginsky, 2018). The few targeted proteomic studies conducted so far to

identify markers of important traits have focused on crops such as potato (Chawade *et al.*, 2016), apple (Buts *et al.*, 2014), grapevine (Riebel *et al.*, 2017) and tomato (Martin *et al.*, 2016; Mata *et al.*, 2018). Some have examined gluten profile (Bromilow *et al.*, 2016; Bose *et al.*, 2019) but even fewer have dealt with forest species. Although selected reaction monitoring (SRM) and its variants are the current gold standard for quantitative estimation of proteins, the recently data-independent acquisition (DIA) method is increasingly being used for targeted proteomics. Unlike existing alternatives, DIA extracts specific information from previously acquired data (Meyer and Schilling, 2017). This method has been used in combination with proteotypic peptides to identify peptide markers of resistance to *Peyronellaea pinodes* in pea (Castillejo *et al.*, 2020). The use of proteotypic peptides for protein quantification recently proved more accurate than methods based on algorithms (usually on the intensity of the strongest ion peaks) (Escandon *et al.*, 2021).

As shown here, the shotgun technique, in combination with proteotypic peptides extracted from previously acquired data, has a great analytical potential. While not a targeted proteomic strategy proper, this approach allows one to select peptides and proteins closely associated with specific traits. This requires processing a dataset compiled from a properly designed and conducted experiment (viz., one using a large enough number of replicates or individuals). In this work, we compiled a list of peptides and proteins potentially useful as putative markers of drought tolerance in *Q. ilex* that are briefly discussed in biological terms below. The proteins in the

marker panel were differentially represented among populations, with the greatest numbers of changes found in the Huelva population, followed by Seville, Granada and Cadiz. Many of the selected proteins are involved in synthesis or degradation processes, or, to a lesser extent, in metabolic processes such as carbohydrate metabolism, photosynthesis and other metabolic reactions. Stress response, redox and secondary metabolism proteins were also well represented.

Changes in synthesis and degradation proteins under stressing conditions such as drought can be interpreted as a mechanism of adaptation through installation of the translational apparatus and protein synthesis by recycling available amino acids in plants through protein degradation. Thus, plants respond to drought by synthetizing protective proteins and repairing or degrading damaged proteins (Vaseva *et al.*, 2012). Considering the general changes observed in the proteome in response to drought, synthesis (ribosomal and transcription) was the most represented group of proteins showing qualitative and quantitative changes, followed by folding and degradation category. Many of the proteins selected as putative markers here are involved in synthesis processes; such is the case, for instance, with translation initiation factor, zinc finger protein VAR3, RNA-binding protein, and methionyl-tRNA synthetase. The marker panel also included folding and degradation proteins such as the chaperones calreticulin and GrpE protein, and serine protease subtilisin. 2DE-MSMS proteomic analysis previously revealed a similar response involving some of the previous proteins in *Q. ilex* (Valero-Galvan *et al.*, 2013; Simova-Stoilova *et al.*, 2015) and *Q. robur*

(Sergeant *et al.*, 2011) under drought, and suggested active metabolic adjustment to stress.

By contrast, degradation of starch in response to stress has been often associated with improved tolerance and potentially limited photosynthesis (González-Cruz *et al.*, 2012; Cuellar-Ortiz *et al.*, 2008). Sugars resulting from starch degradation, and other derivative metabolites, help plants grow under stress and function as osmoprotectants and compatible solutes to mitigate the adverse effects of stress (Krasensky and Jonak, 2012), as found in droughted *Q. robur* (Sergeant *et al.*, 2011). Although environmental factors are known to have strong effects on starch synthesis, their regulatory mechanisms remain unclear (Thalmann and Santelia, 2017). Some studies reported increased starch accumulation under stress, mainly in response to high salinity or cold (Kaplan and Guy, 2005; Yin *et al.*, 2010; Skirycz *et al.*, 2010). In this work, two proteins of carbohydrate metabolism of the marker panel (viz., granule-bound starch synthase 1, which is chloroplastic/amyloplastic, and the glycosyl hydrolase family protein with chitinase insertion domain) were found at increased levels in, mainly, the Seville population. However, several starch degradation proteins not selected as putative markers were significantly increased in response to drought in some of the experimental conditions studied, including phosphoglucan water dikinase, alpha-glucan phosphorylase and alpha-amylase. Although apparently contradictory, this response may be related to the presence of different types of starch, whether permanent or transitory, and that of isoforms involved in their synthesis and mobilization (Wang *et al.*, 2006;

Thalmann and Santelia, 2017; Prathap and Tyagi, 2020). On the other hand, the photosynthetic machinery was seemingly unaffected; in fact, only a few photosynthesis proteins exhibited any changes and only one (chlorophyll a-b binding protein) was included in the marker panel. This result is consistent with those of physiological studies on *Q. ilex* populations under severe drought in Seville, Granada and Cadiz which exhibited no significant changes in photosynthetic pigments (San-Eufrasio *et al.,* 2020).

The broadest group of proteins and derived peptides selected as putative markers consisted of redox (2-alkenal reductase NADP-dependent, short-chain alcohol dehydrogenase A, disulfide-isomerase) and stress response proteins (endoplasmin, dehydrin, senescence/dehydration-associated protein and aldo-keto reductase). Some were closely associated with drought in several studies on the genus *Quercus* (Echevarria-Zomeno *et al.,* 2009; Sergeant *et al.,* 2011) or with biotic stress caused by *Phytophthora cinnamomi* (Sghaier-Hammami *et al.,* 2013). Furthermore, a representative number of redox proteins not included in the marker panel have been identified as being increased to a greater or lesser extent in some of the conditions studied in response to drought, including glutathione S-transferase, glutathione peroxidase, thioredoxin, peroxidase, superoxide dismutase, lypoxygenase, among others. Our marker panel also included two enzymes involved in the shikimate-phenolic biosynthetic pathways, namely: chalcone synthase (CHS) and 3-phosphoshikimate 1-carboxyvinyltransferase. One of the potential roles of phenolic compounds is to scavenge harmful reactive oxygen species (Sharma *et al.,* 2019).

Consistent with our results, *Q. ilex* (Nogués *et al.*, 2013; San-Eufrasio *et al.*, 2020) and other *Quercus* spp. (Jafarnia *et al.*, 2018; Ghanbary *et al.*, 2020) were previously found to exhibit increased total levels of phenolics.

Finally, transport proteins such as the water channel proteins aquaporins have been associated with plant tolerance of biotic and abiotic stresses, to which they respond by regulating the movement of water and small molecules through plasma membranes and vacuoles (Li *et al.*, 2015). Based on a proteomics strategy involving the identification of proteotypic peptides, some transport proteins have been proposed as markers of tolerance to drought (Castillejo *et al.*, 2016) and resistance to *Ascochyta* blight (Castillejo *et al.*, 2020) in pea. The proteins were assumed to induce signaling and transport processes as mechanisms to maintain homeostatic equilibrium and cope with stress. The proposed putative markers included the importin subunit alpha, aquaporin and mitochondrial fission 1 protein A, although other transport and signaling proteins, such as 14-3-3 like protein, lipocalin, outer envelope pore protein (OEP), voltage dependent anion-selective channel (VDAC) and translocase of chloroplast 90 protein, were also more represented under drought in some of the experimental conditions in this study.

Despite it is outside the scope of this work, based on our results a clear distinction in the response to drought among the populations studied cannot be postulated, for which a greater number of populations covering a wider area must be included. However, attending to the high number of the proteins

that showed changes in the Huelva population also observed in Seville, we could speculate on a similar response pattern to drought in both, perhaps due to geographic proximity. The fact that the proteomic profile of Cadiz is different from the rest may also have a geographical explanation as has already been described by Fernandez i Marti *et al.* (2018), suggesting that the Guadalquivir Valley has played an important role in determining population divergence. To the authors' knowledge this is the first study aimed to identify proteins and derived peptides as putative markers of drought tolerance for a forest species such as *Q. ilex*. Such markers may be useful with a view to selecting drought tolerant genotypes or individuals.

5. Conclusions

Methodologically, the proposed targeted strategy is aimed at identifying peptides associated with the response of *Q. ilex* to drought stress. As a supplement to shotgun analysis, using proteotypic peptides in addition to proteins allows putative markers enabling the identification of specific phenotypes such as that best resisting drought to be selected. Our methodological workflow consisted on selecting those consistent and confidence proteins, whose proteotypic peptides were used for quantification. Of them, 46 peptides showed significant changes in response to drought stress in the same way that the protein they come from, which were proposed as putative markers.

Biologically, the results suggest that plants possess effective protective mechanisms for adaptation to drought through water loss prevention, and

protein protection and detoxification. However, small differences in response mechanisms may result in plant survival or adaptation to extreme conditions depending on the particular population or individual. As can be inferred from the composition of the marker panel, *Q. ilex* seedlings from four different populations responded differentially to drought, with the greatest number of changes being observed in the Huelva and Seville populations. Only two proteins (viz., the protease subtilisin and chaperone GrpE protein) were increased to a similar extent in three of the four populations. These proteins should be validated as biomarkers of drought tolerance in *Q. ilex* with further testing.

Our study constitutes a step forward in the molecular elucidation of this forest species. Advances in molecular techniques including omics, in combination with physiological studies, can be expected to allow especially tolerant or resilient genotypes or individuals under stress conditions such as those propitiated by a climate change scenario to be selected in reforestation programs.

6. Supplementary Materials

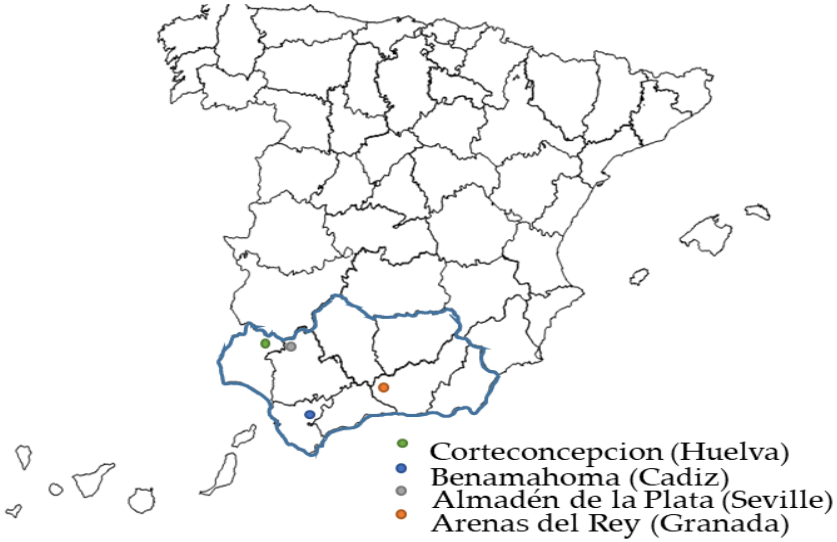


Figure S1: Location of all Andalusian *Q. ilex* provenances used in this study. Andalusia is delimited by the blue outline.

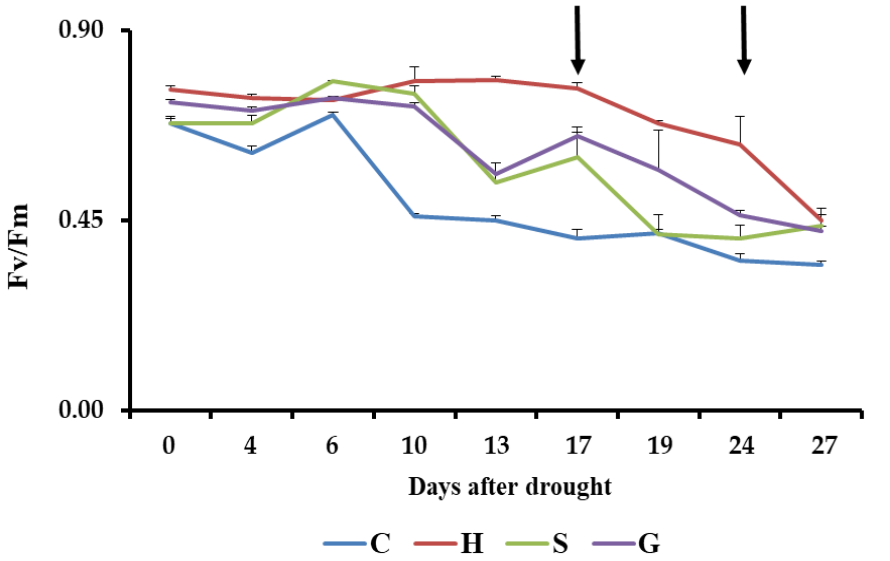
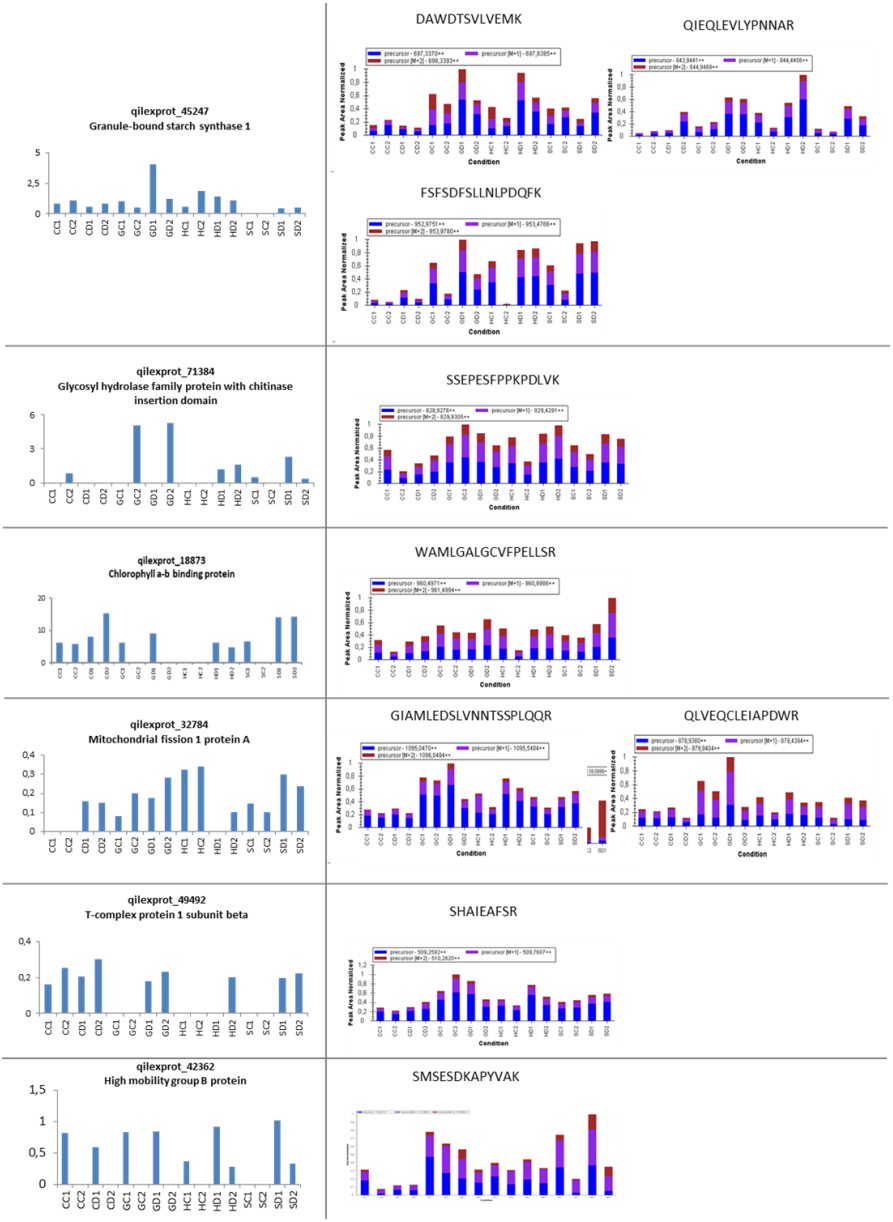


Figure S2. Measurements of quantum yield of photosystem II (Fv/Fm) in dark-adapted leaves of *Q. ilex* of Andalusian provenances (Cadiz, C; Granada, G; Huelva, H; Seville, S) under drought conditions throughout the experiment. Values are means ± standard errors (SE). Arrows indicate the sampling times when the mean of leaf fluorescence had decreased by 20% after 17 days and 40% after 24 days relative to control seedlings.

Figure S3: Visual damage symptoms in four Andalusian *Q. ilex* provenances (C, Cadiz; H, Huelva; S, Seville; G, Granada; the letters following C, H, S or G denotes treatment: D, drought; C, control) 25 days after drought treatment. Control seedlings did not show damage symptoms throughout the experiment. In contrast, some seedlings did show clear damage symptoms related to drought. Only asymptomatic seedlings were used in this study.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SSEPESFPPKPDLVK
qilexprot_18873
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Peak Area Normalized
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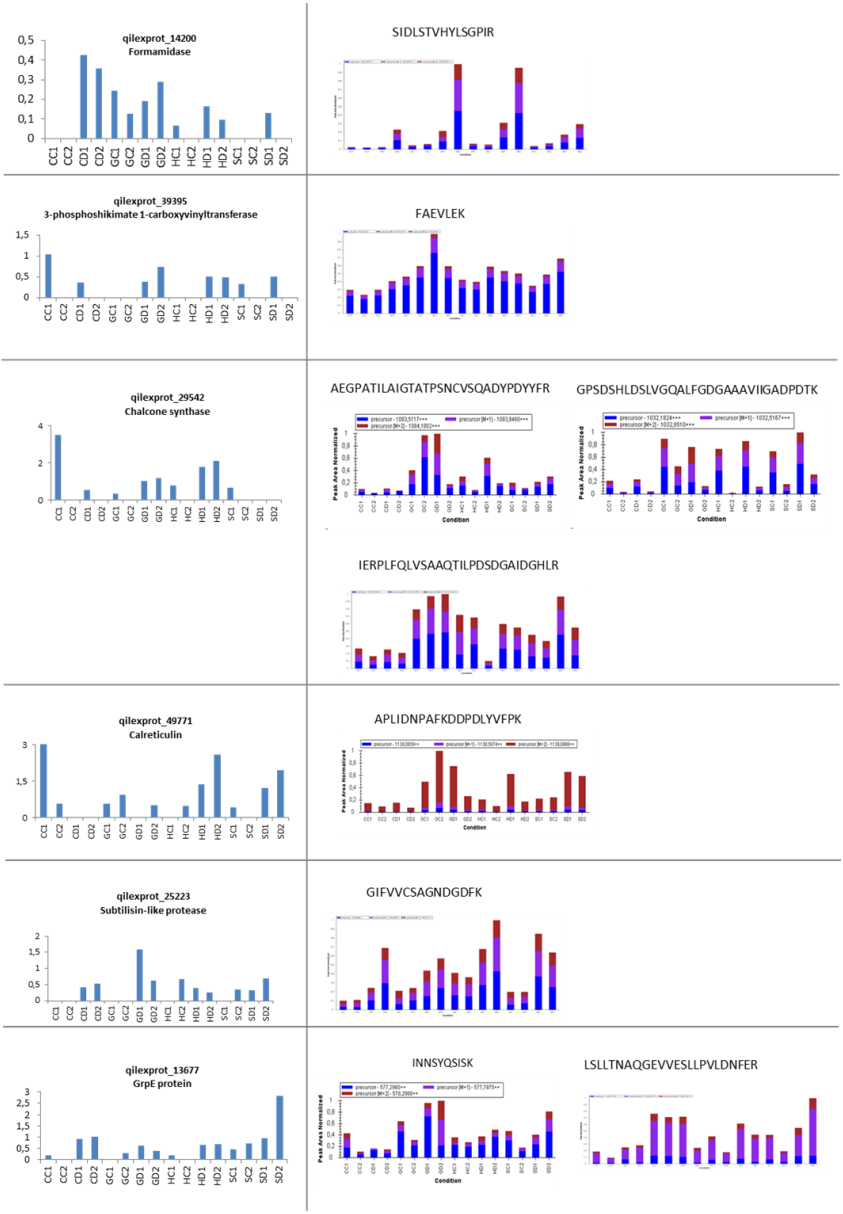
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qilexprot_25223
Subtilisin-like protease
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qilexprot_13677
GrpE protein
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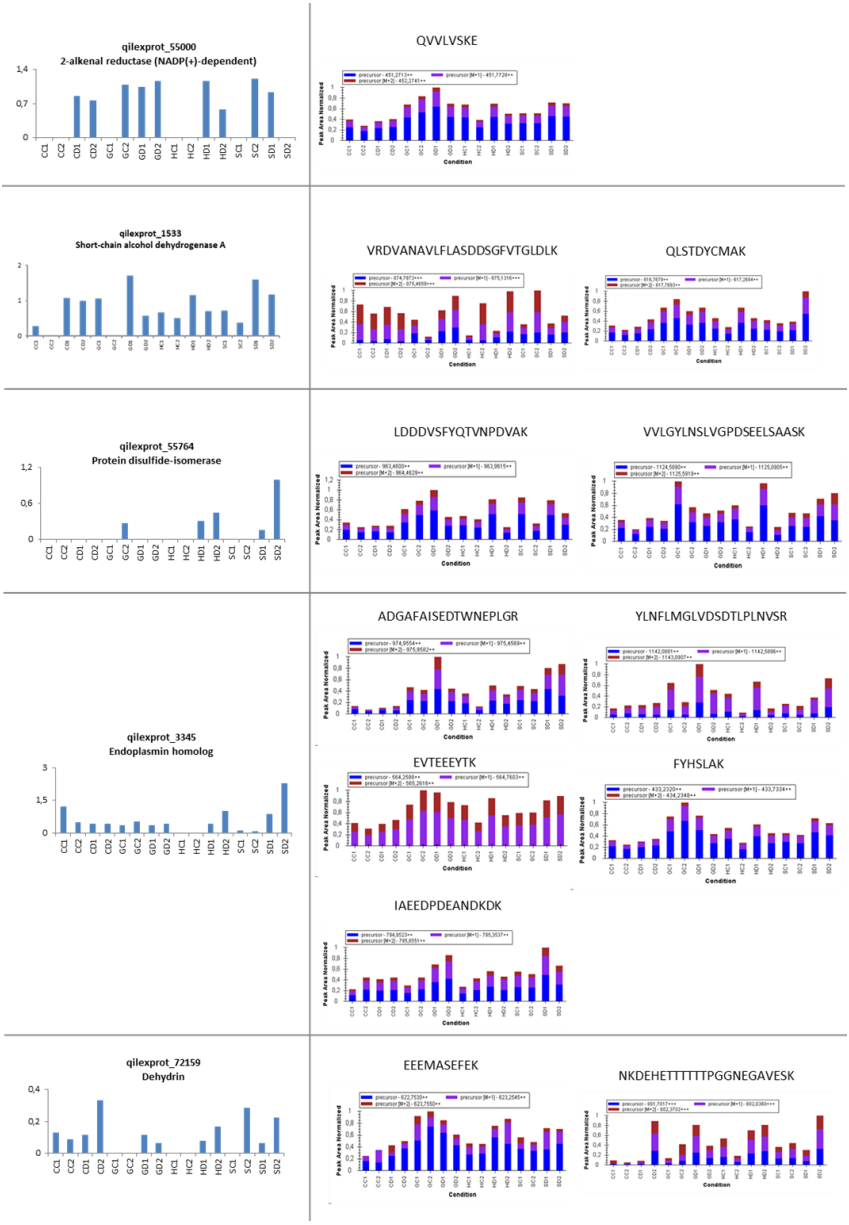
Peak Area Normalized
Condition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qilexprot_55764
Protein disulfide-isomerase
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qilexprot_3345
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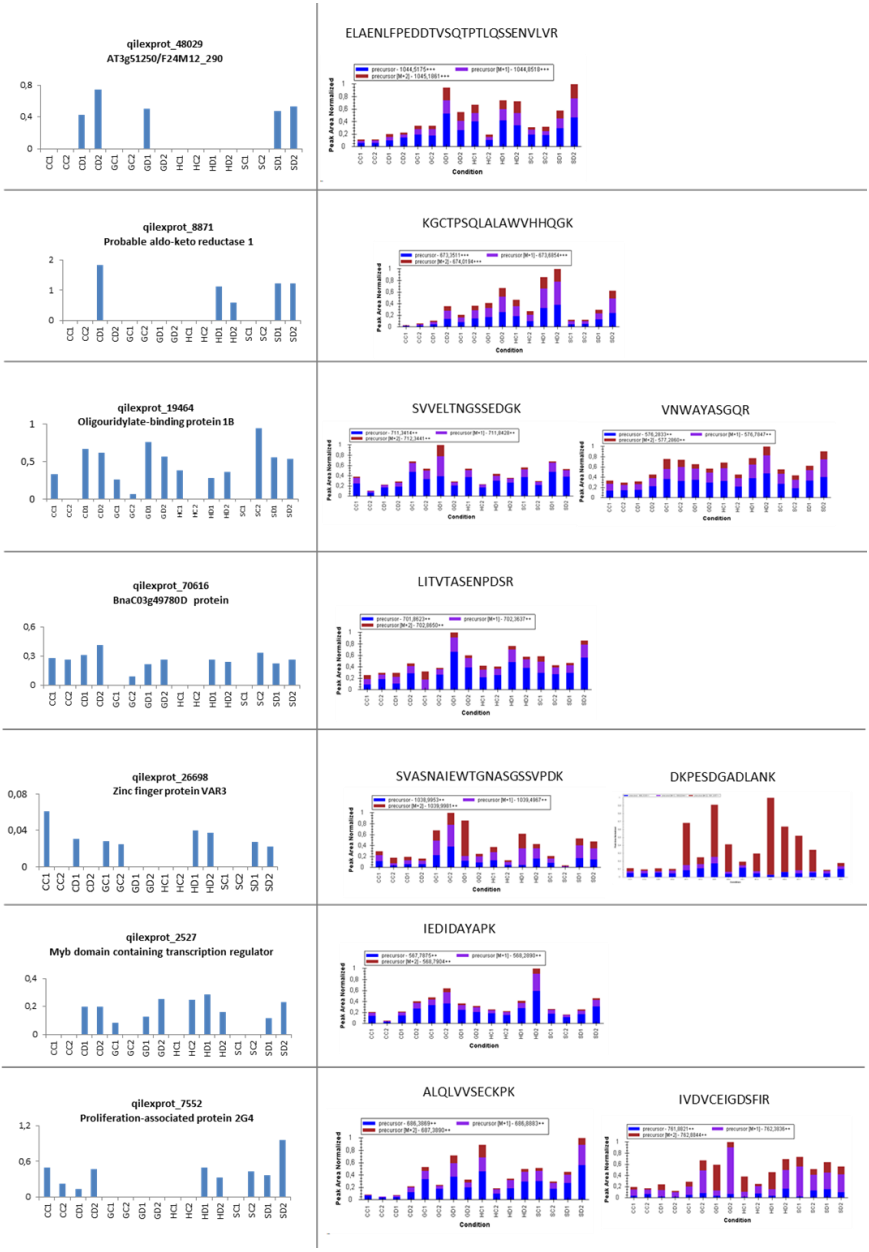
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qilexprot_70616
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Zinc finger protein VAR3
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Condition

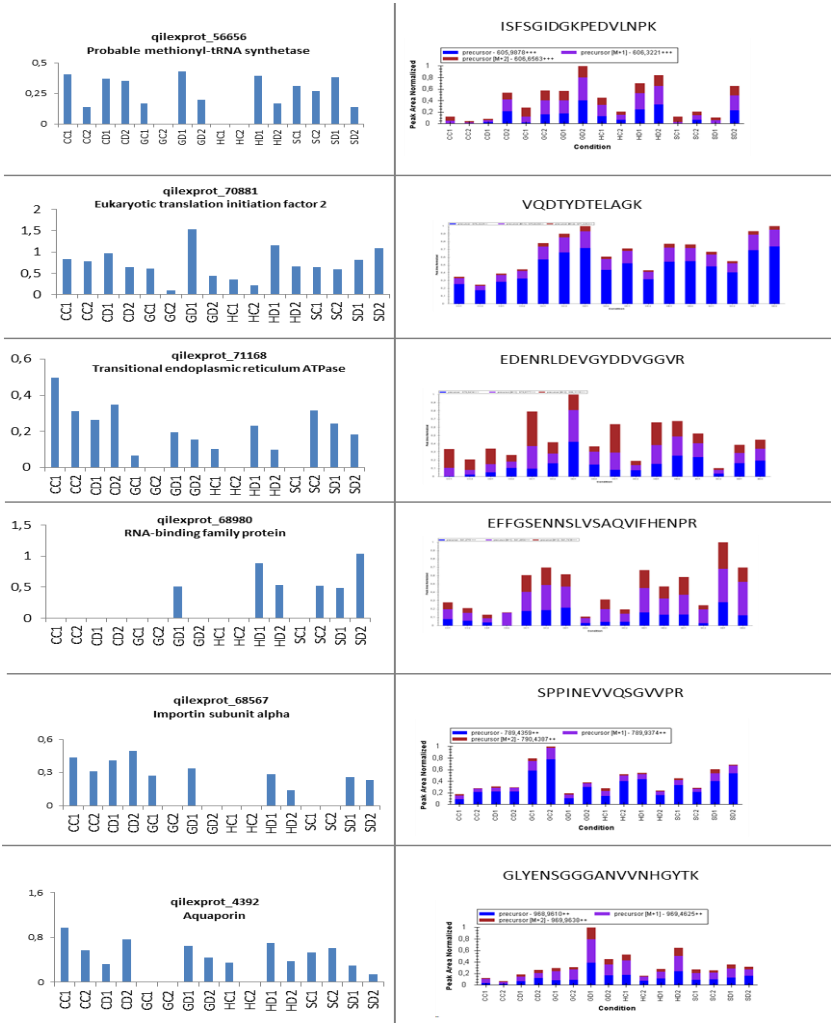

Figure S4: Quantitative protein (left) and abundance of precursor ions (right).

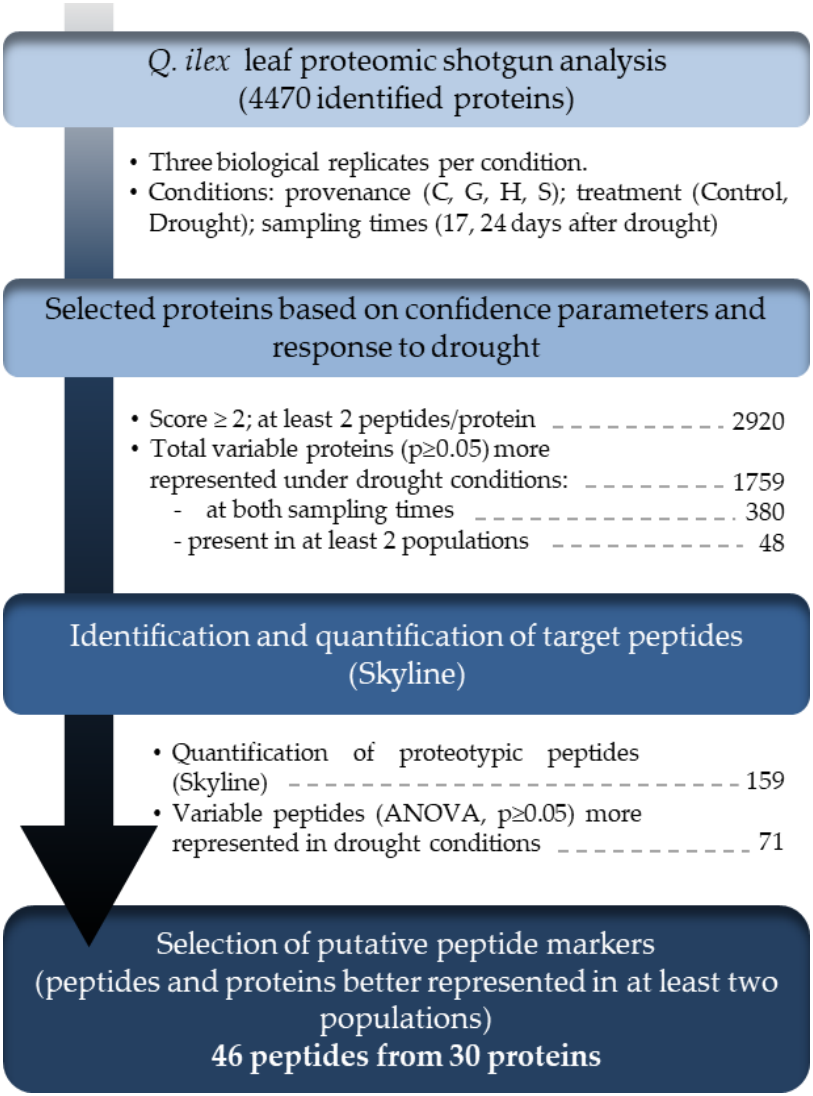

Figure S5: Schematic workflow for selection of putative tolerance drought markers in *Q. ilex* seedlings by targeted post-acquisition proteomic analysis. C, Cadiz; G, Granada; H, Huelva; S, Seville.

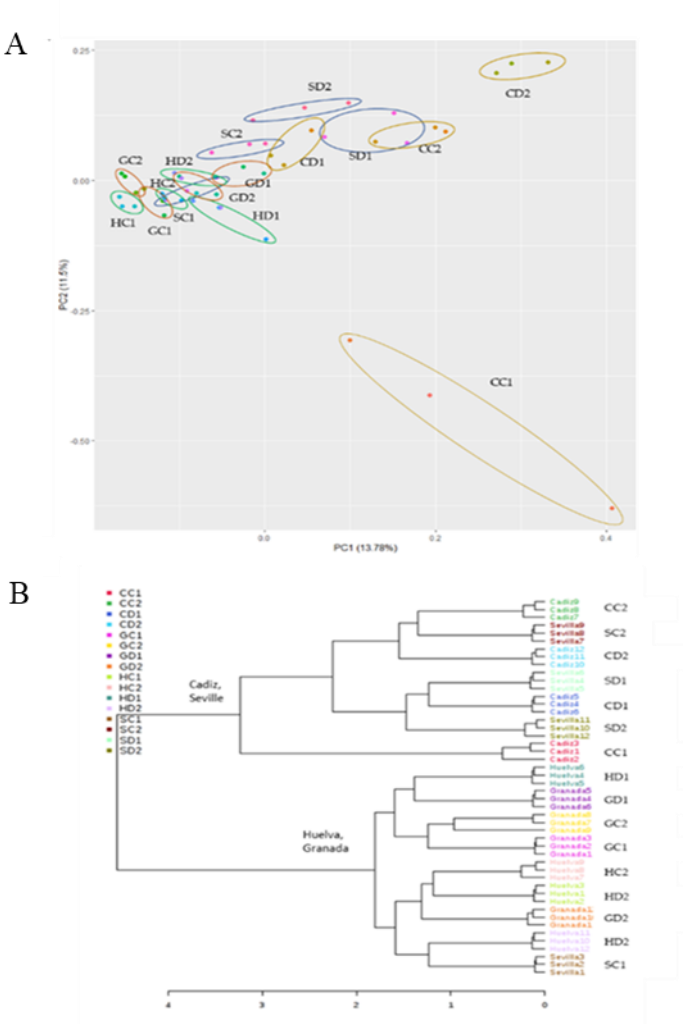

Figure S6: multivariate statistical analysis (PCA) of the entire dataset (A). Dendrogram shows hierarchical clustering of experimental conditions (B). C, Cadiz; G, Granada; H, Huelva; S, Seville. The letters following C, G, H, or S denotes treatment (D, drought; C, control), the numbers sampling time (1, 17 days; 2, 24 days).

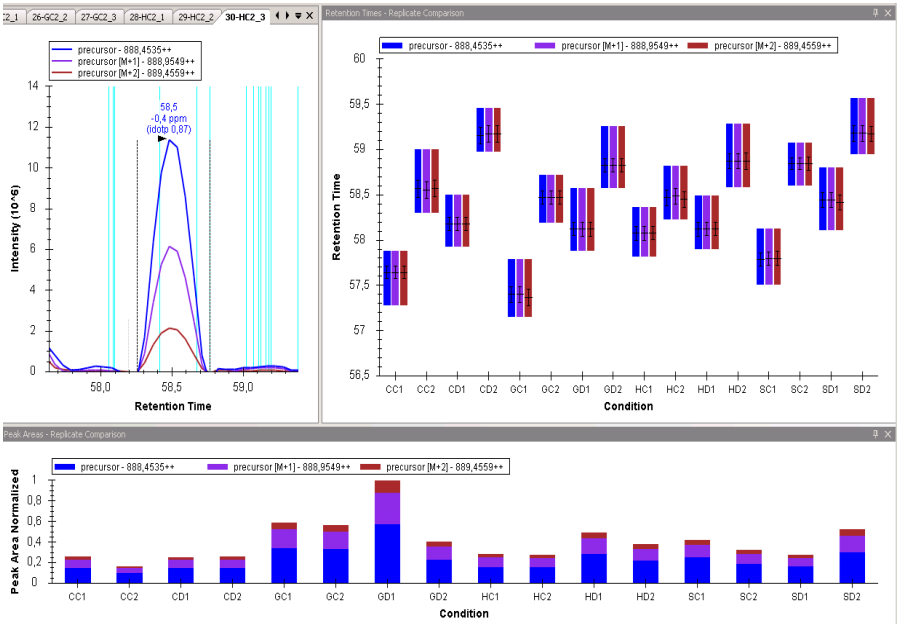

Figure S7. Representative MS1 fragment ion, retention times (RT) and normalized peak area of a target precursor ion given by the software Skyline.

Table S1: Locations and environmental conditions of the four *Q. ilex* Andalusian populations.

Locations	MASL (m)	Coordinates (ETRS89)	Tmax (ºC)	Tmin (ºC)	P (mm)
Benamahoma, Cadiz (C)	649	36°45'N, 5°27'W	24.9	9.8	1263.6
Corteconcepcion, Huelva (H)	369	37°55'N, 6°28'W	26.3	5.5	945.6
Almadén de la plata, Seville (S)	482	37°52'N, 6°28'W	26.4	9.5	722.1
Arenas del Rey, Granada (G)	892	36°57'N, 3°54'W	24.7	11.5	489.3

Altitude (meters above sea level (MASL)), coordinates ETRS89, Minimal (Tmin) and maximum (Tmax) average temperature, and average annual rainfall (P).

Chapter IV

Effect and response to combined *Phytophthora cinnamomi* and drought in *Quercus ilex* subsp. *ballota* [Desf.] Samp. seedlings from three contrasting Andalusian populations

Bonoso San-Eufrasio, María Ángeles Castillejo, Mónica Labella-Ortega, Francisco J. Ruiz-Gómez, Rafael M Navarro-Cerrillo, Jesús V. Jorrín-Novo, María-Dolores Rey. Frontiers in Plant Science. Under evaluation.

Abstract

Quercus ilex L. is the dominant species in the Mediterranean forest and agrosilvopastoral ecosystem "*dehesa"*. Currently, this forest species is threatened by natural and anthropogenic agents, but especially by the decline syndrome, which is caused by *Phytophthora cinnamomi* and drought periods. Although the morphological and physiological responses of *Q. ilex* to combined stress (*P. cinnamomi* and drought) have been examined already, little is known at the molecular level. In this work, we studied the effect and response of 8-month seedlings from three contrasting Andalusian populations (Seville, Granada and Almeria) to the individual and combined stress of *P. cinnamomi* and drought in morphological, physiological, biochemical and proteomic terms. Whereas seedling damage (leaf chlorosis and necrosis) and mortality was greater under the combined stresses in the three populations, the effect of each individual stress was population-dependent. Seedling survival differed significantly among populations and treatments. Similarly, the decrease in leaf fluorescence, photosynthetic activity, stomatal conductance and transpiration rate observed was greater in the presence of both stresses, the three populations responding similarly to drought and *P. cinnamomi*. Biochemical and proteomic analyses of undamaged seedlings from the two most markedly contrasting populations (Seville and Almeria) revealed the absence of significant differences in the contents in photosynthetic pigments, amino acids and phenolics among treatments. By exception, the contents in sugars, starch and flavonoids were higher in the Seville population than in the Almeria population. The Seville and Almeria populations exhibited changes in protein profile in response to

the different treatments, with 83 variable proteins in the former population and 223 in the latter. The proteins concerned belonged to 16 different functional groups the best represented among which were folding, sorting and degradation; carbohydrate metabolism; energy metabolism; amino acid metabolism, ROS scavenging and secondary metabolism. Most groups exhibited up-accumulation but energy metabolism proteins were down-accumulated. Although no treatment-specific response was observed in any functional group, differences in response intensity were especially marked under the combined effect of the two stresses. The following variable proteins are proposed as putative markers for resilience in *Q. ilex*: aldehyde dehydrogenase, glucose-6-phosphate isomerase, 50S ribosomal protein L5 and alpha-1,4-glucan-protein synthase [UDP-forming].

Keywords: Holm oak, decline syndrome, climate change, combined stress, proteomics, molecular markers

1. Introduction

Holm oak (*Quercus ilex* L.) is the dominant species in Mediterranean basin forests and also in the long-established agrosilvopastoral oak open woodlands called *dehesas* in Spain and *montados* in Portugal (Ruiz de la Torre, 2006; De Sampaio *et al.*, 2013). This species possesses a high environmental and ecological importance (Guzmán *et al.*, 2016). Thus, it adapts well to arid and semi-arid regions, where it plays a key biological role against desertification (Quero *et al.*, 2006). In recent decades, however, increasing tree defoliation and mortality in large areas of the western Iberian Peninsula are endangering holm oak forests along the Mediterranean basin (Brasier, 1996; Jung *et al.*, 2000; Natalini *et al.*, 2016; Sánchez-Cuesta *et al.*, 2021). Tree mortality is associated with both natural and anthropogenic factors such as overexploitation and poor regeneration or livestock management, and also with the severe effect of external biotic and abiotic factors such as attack by soilborne pathogens, extreme temperatures, heavy rainfall episodes and extended drought periods, which in combination result in so-called "oak decline syndrome" (Brasier, 1992; De Sampaio *et al.*, 2013; Surová *et al.*, 2017; Corral-Ribera *et al.*, 2018).

Holm oak decline is a complex syndrome usually triggered by extreme climate events such as drought and high temperatures or invasive pathogens such as oomycetes (Sánchez *et al.*, 2002; Valero-Galván *et al.*, 2013; Sghaier-Hammami *et al.*, 2013; Ruiz-Gómez *et al.*, 2018). However, there is solid evidence that drought, and root rot by effect of *Phytophthora cinnamomi* Rands., are the two main factors triggering strong tree death

episodes (Brasier, 1996; Sánchez *et al.*, 2002; Corcobado *et al.*, 2014, 2017; Ruiz-Gómez *et al.*, 2018, 2019; Gea-Izquierdo *et al.*, 2021). *Phytophthora cinnamomi* is one of the worst invasive alien pathogens around the world (Burgess *et al.*, 2017), its spectrum of hosts including more than 5000 different species (Hardham, 2005). This oomycete is heterothallic (i.e., it has two different mating types, of which only Type A2 is present in the Iberian Peninsula). The pathogen reproduces asexually by sporulating motile zoospores that can be carried by soil water to find new roots and spread easily under the typical conditions of Mediterranean climate (viz., short episodes of heavy rainfall, intermittent flooding and heavy run-off).

Previous studies have shown drought to increase the susceptibility of *Q. ilex* seedlings to *P. cinnamomi* root rot (Corcobado *et al.*, 2014, 2017; Ruiz-Gómez *et al.*, 2018). This finding is supported by the fact that plants are weakened under environmental cues (Agrios, 2005; Desprez-Loustau *et al.*, 2006). The combined effects of biotic and abiotic stresses on *Q. ilex* seedlings have so far been studied in phenotypic, physiological and biochemical terms (Corcobado *et al.*, 2014; Ruiz-Gómez *et al.*, 2018, Colangelo *et al.*, 2018). Thus, the presence of *P. cinnamomi* is known to trigger unspecific defense responses such as accumulation of phenolics, thickening of cell walls and callose accumulations (Redondo *et al.*, 2015; Ruiz-Gómez *et al.*, 2015). Under drought, these conditions result in substantial changes in biomass allocation such as a decrease in root biomass and also in physiological activity-related parameters such as CO_2 assimilation, stomatal conductance and leaf fluorescence (Ruiz-Gómez *et*

al., 2018). These changes must no doubt reflect at the molecular level, but how it does remains largely unknown to date. Recently, Ruiz-Gómez *et al.* (2018) examined changes in a *Q. ilex* population in Arenas del Rey (Granada, Andalusia, Spain) under stress from both *P. cinnamomi* and drought. However, the high inter- and intra-population variability of this species (San-Eufrasio *et al.*, 2020) requires comparing various populations in order to better elucidate the response of holm oak to the conditions causing the decline syndrome. Also, molecular studies using the most recent tools available for this purpose could be useful to gain further insight into variability in this species, and also to help identify key genes and gene products involved in the response to the syndrome (Rey *et al.*, 2019).

In this work, we studied the effect of exposure of *Q. ilex* to drought and attack by a pathogen (*P. cinnamomi*) individually and in combination from a physiological, biochemical and proteomic perspective. For this purpose, we examined the response to and tolerance of *P. cinnamomi* and drought in three contrasting Andalusian *Q. ilex* populations (Seville, Granada and Almeria). Elucidating the molecular mechanisms behind resilience to both stresses in *Q. ilex* from physiological and molecular data allowed us to put forward several putative gene markers for use in breeding actions in the framework of conservation and afforestation programmes.

2. Materials and methods

2.1. Plant material

Acorns were collected by staff of the Department of Forestry Engineering of the University of Cordoba from three different *Q. ilex* populations in Andalusia, namely: Almaden de la Plata, Seville (Se); Arenas del Rey, Granada (Gr); and Sierra Maria, Almeria (Al) (Table 1).

Table 1. Location and environmental features of the three Andalusian *Q. ilex* populations used in this study. Altitude (Meters Above Sea Level–MASL), coordinates ETRS89, average temperature of the coldest month (*Tmin*, ºC), average temperature of the warmest month (*Tmax*, ºC), and average annual rainfall (*P*, mm) (Navarro-Cerrillo *et al.*, 2018).

	Andalusia location	**Altitude**	**Coordinates**	***Tmax***	***Tmin***	***P***
More Eastern	Sierra María (Al)	890	37°42'N 2°07'W	18.0	5.8	411.0
↓	Arenas del Rey (Gr)	892	36º57'N, 3º54'W	24.7	11.5	489.3
More Western	Almaden de la plata (Se)	482	37º52'N, 6º05'W	26.4	9.5	722.1

Healthy acorns were selected after surface cleaning with 5% HCl and suspension in water, floating acorns being discarded. In January 2019, acorns were pre-germinated in a wet bed and sown in black plastic pots (3 L, 14.5 × 14.5 × 22 cm) containing perlite from Gramoflor GmbH and Co. (Vechta, Germany). Pots were placed in a temperature-controlled greenhouse at 25 ± 7°C at 60 ± 10% relative humidity located in Cordoba (Andalusia, Southern Spain; 37°54′46″ N, 4°43′15″ O). The experiment was started in October

2019, when seedlings were ~15 cm tall. Seedlings were irrigated every two days with tap water (200 ml) and once a week with Hoagland nutrient solution up to the start of the experiment (Hoagland and Arnon, 1950).

2.2. Experimental design and treatments

The experimental design (Figure S1) encompassed four different treatments, namely: (1) irrigation to field capacity in the absence of *P. cinnamomi* (control treatment); (2) no irrigation and no *P. cinnamomi* (drought treatment); (3) irrigation and *P. cinnamomi* inoculation (inoculation treatment); and (4) *P. cinnamomi* inoculation and no irrigation (combined treatment). The treatments were performed as described by Ruiz-Gómez *et al.* (2018) and Sghaier-Hammami *et al.* (2013). The experiment was conducted according to a completely randomized design with 20 seedlings per treatment (80 seedlings per population) and a duration of 35 days.

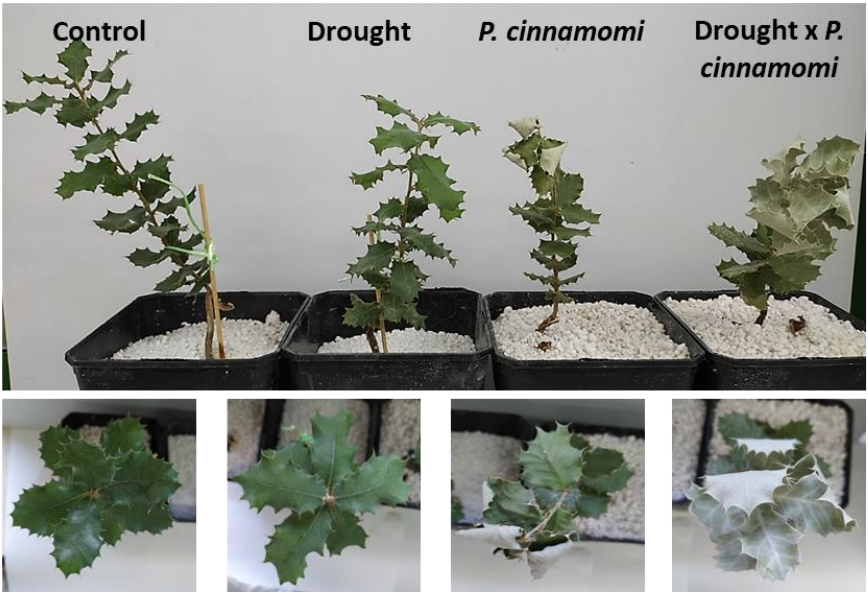

Figure 1. Visually identified damage in *Q. ilex* seedlings from the Seville population. Treatments: control (C), drought (D), *P. cinnamomi* (I) and drought × *P. cinnamomi* (D×I).

Phytophthora cinnamomi (P90) previously isolated from *Q. ilex* roots in Puebla de Guzmán (Huelva, Andalusia, Spain), was reactivated by using root cuts inoculated in a PARPBH selective medium containing piramicin, amplicilin, rifamycin, pentachloronitrobenzene, benomyl and hymexazol (Jeffers and Martin, 1986; Ruiz-Gómez *et al.*, 2018). The inoculation protocol used was that of Ruiz-Gómez *et al.* (2018) except that the root system was brought into contact with the pathogen by immersion in Carror–Agar (CA) liquid inoculum (Sghaier-Hammami *et al.*, 2013) at a concentration of 39 chlamydospores/µL (Ruiz-Gómez *et al.*, 2018). Control

seedlings were also immersed in CA liquid inoculum but containing no *P. cinnamomi*. After inoculation, seedlings were transplanted into pots filled with fresh perlite. All pots were flooded for 48 h, excess water being removed before the experiment. In the drought treatment, water was withheld according to San-Eufrasio *et al.* (2020). Control and inoculated seedlings were irrigated to field capacity throughout. The presence of *P. cinnamomi* in the root system was checked on days 19 and 32 by using fine roots from each seedling as described by Ruiz-Gómez *et al.* (2018). Briefly, three pieces of fine roots per seedling (< 2 mm thick, ~1 cm long) were randomly selected and immersed in 70% ethanol, washed in sterilized–de-ionized water, and placed in 9-cm Petri dishes containing PARPBH selective medium. The pathogen was identified morphologically by conventional light microscopy (Erwin and Ribeiro, 1996).

2.3. Perlite Water Content and Matric Potential

Both the perlite water content (PWC, %) and the matric potential (Ψ_m, kPa), were estimated according San-Eufrasio *et al.* (2020) throughout the experiment. The former parameter was calculated as follows:

$$PWC_t\ (\%) = [PWW_t - (PDW/PWW_0) - PDW] \times 100$$

Where PWW denotes pot wet weight; PDW pot dry weight; and *t* time, in days, with 0 corresponding to the initial, maximum value.

2.4. Damage Symptoms and Seedling Mortality

Damage symptoms (viz., leaf chlorosis and wilting) resulting from the presence of *P. cinnamomi* and/or drought were evaluated visually in all seedlings and confirmed by taking photographs of all seedlings with a digital camera. When all leaves exhibited severe drought symptoms (viz., a dry-yellow appearance throughout) and quantum yield of Photosystem II (Qy) near 0, the number of dead seedlings was also recorded.

2.5. Physiological measurements

Relative leaf water content (RLWC) was calculated on day 32 from fresh (FW), turgid (TW) and dry (DW) weights as previously described by San-Eufrasio *et al.* (2020). RLWC (%) was calculated as [(FW − DW)/(TW − DW)] × 100. The quantum yield of Photosystem II (Qy) was measured with a FluorPen FP100 portable leaf fluorimeter from Photon Systems Instruments (Drasiv, Czech Republic) at 3-day intervals throughout the experiment (San-Eufrasio *et al.*, 2020). Measurements were always made on the same three youngest full expanded leaves in each seedling, using 3 seedlings per treatment per population (36 seedlings per measurement) throughout the experiment. All measurements were made in the early morning, when the leaves were adapted to darkness throughout the night according to Strasser *et al.* 2000. Net photosynthesis (A, μmol CO_2 m^{-2} s^{-1}), stomatal conductance (Gs, mol H_2O m^{-2} s^{-1}), transpiration rate (Tr, mmol H_2O m^{-2} s^{-1}) and F_v'/F_m' were measured on a fully expanded leaf in 5 seedlings per treatment per population, using a portable infrared CO_2 gas analyser (IRGA, LiCor Li6400XT, Li-Cor, Inc.; Lincoln, NE, USA) once a

week. All measurements were made between 11:00 and 14:30 UTC (Universal Time Coordinates).

2.6. Photosynthetic Pigment and Metabolite Content

Photosynthetic pigments and metabolites were quantified in asymptomatic leaves from the Se and Al populations. Measurements were made on day 32, when leaf fluorescence had decreased by 20%, 30% and 35% in the drought, inoculation and combined treatment, respectively, relative to the control seedlings (López-Hidalgo *et al.*, 2021). Three biological replicates per treatment per population were used for this purpose. Leaves from each seedling were collected, washed with distilled water and immersed in liquid nitrogen prior to grinding in a pre-cooled mortar. Leaf tissue (50–70 mg fresh weight) was extracted with 80% (v/v) ethanol, the crude extract being centrifuged and the resulting supernatant collected to quantify photosynthetic pigments (chlorophyll a and b, and carotenoids) and metabolites (total free amino acids, soluble sugars, total phenolics and total flavonoids) on an Evolution 201 UV–Vis spectrophotometer from Thermo Fischer Scientific (Waltham, MA, USA) (López-Hidalgo *et al.*, 2021). The resulting pellet was extracted with perchloric acid to quantify starch (López-Hidalgo *et al.*, 2021) by measuring the absorbance at 595 nm of the supernatant against a hydrolysed starch standard (Viles and Silverman, 1949; Rose *et al.*, 1991). The absorbance of the supernatants containing chlorophyll a, chlorophyll b and carotenoids was recorded at 470, 649 and 664 nm, respectively, and used to calculated the respective contents, all in micrograms per millilitre, as follows: chlorophyll a = $13.36 \cdot A_{664} - 5.19 \cdot A_{649}$;

chlorophyll b = 27.43·A_{649} – 8.12·A_{664}; carotenoids = [(1000·A_{470} – 2.13·chlorophyll a – 97.63·chlorophyll b)/209] (Lichtenthaler, 1987). The absorbance of the supernatants containing total free amino acids, soluble sugars, total phenolics and total flavonoids was measured at 450, 595, 750 and 415 nm, respectively, using a standard of (1:1) proline–glycine, glucose, gallic acid and quercetin, respectively. The crude extract containing total free amino acids, soluble sugars, total phenolics and total flavonoids was mixed thoroughly with (2:1 v/v) ninhydrin reagent (Starcher, 2001), (1:16 v/v) anthrone reagent (Thayermanavan and Sadasivam, 1984) and 10% (1:2 v/v) Folin–Ciocalteu reagent, respectively, followed by addition of (3:8 v/v) sodium carbonate (Viles and Silverman, 1949), 10% (10:1) (v/v) aluminium chloride–1 M potassium acetate and (22:35 v/v) methanol (Mammnen and Daniel, 2012), respectively.

2.7. Proteomic Analyses

Proteomic runs were also performed on the Se and Al populations. Protein extracts were obtained from 300 mg of fresh leaf tissue from asymptomatic seedlings, using the TCA/acetone-phenol protocol (Wang *et al.*, 2006). Proteins were extracted from three independent biological replicates under each set of experimental conditions, and dissolved in a solution containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS {3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulphonate}, 0.5% (w/v) Triton X-100 and 100 mM DTT. Protein contents were quantified with the Bradford method, using bovine serum albumin (BSA) as standard (Bradford, 1976).

2.8. Gel-based proteomic approach (SDS-PAGE)

The proteins extracted from each sample (80 µg of BSA protein equivalent) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gel (Laemmli, 1970), using the Protean II-XL (20 × 20 cm) system from Bio-Rad (Hercules, CA, USA) with a voltage run of 80 V until the dye reached the bottom of the gel. Gels were stained with Coomassie Brilliant Blue R-250 (Neuhoff *et al.*, 1988) and images acquired with a GS-900 Calibrated Densitometer from Bio-Rad. Images were analysed with the software ImageLab™ 5.2.1, also from Bio-Rad, bands being automatically detected and confirmed by visual inspection. The optical density (OD) of each band was normalized against the combination of all bands for each sample. Band molecular masses were calculated by comparing mobilities with those of protein standard markers (SDS Molecular weight standards, Broad range, Bio-Rad).

2.9. Shotgun proteomic analysis (LC-MS/MS)

Extracted proteins (80 µg) were subjected to SDS-PAGE in a 12% polyacrylamide gel Mini-PROTEAN 8.6 × 6.7 cm^2 cell from Bio-Rad. The gel was run at 80 V and stopped when Bromophenol Blue had advanced 0.5 cm into the resolving gel. The gel was stained with Coomassie Brilliant Blue R-250. The resulting unique band was removed with a scalpel, cut into pieces smaller than 1 mm^3 and transferred individually to 1.5 mL tubes for digestion with 12.5 ng μL^{-1} sequencing-grade trypsin from Promega (Madison, WI, USA) (Castillejo *et al.*, 2015). Peptides were desalinated by passage through a C18 resin micro-column from Scharlau (Barcelona, Spain), eluted with

70% acetonitrile (AcN) containing 0.1% trifluoroacetic acid and dried in a speed-vac apparatus. The resulting peptides were resuspended in 4% (v/v) AcN containing 0.25% (v/v) formic acid (FA) (López-Hidalgo *et al.*, 2018; Romero-Rodriguez *et al.*, 2019). Peptides were charged to 0.4 µg/µL by injection into a one-dimensional nano-flow LC–MS/MS system (i.e., liquid chromatography with tandem mass spectrometry) from Thermo Fisher Scientific (Gomez-Gálvez *et al.*, 2020). A monolithic C18 Acclaim PepMap column 15 cm long × 75 µm inner diameter, also from Thermo Fisher Scientific, was used. Peptides were separated at 40 °C in all runs. Solvent A contained 0.1% FA, and solvent B consisted of 80% AcN containing 0.1% FA. Samples were separated by using a gradient from 95% solvent A to 80% solvent B at a controlled flow-rate of 400 nL/min for 120 min. LC was coupled to MS via a nanoelectrospray ionization source. MS analyses were done on a trihybrid Thermo Orbitrap Fusion mass spectrometer from Thermo Scientific operated in the positive ion mode. The specific settings used in the LC–MS/MS analysis are described elsewhere (Castillejo *et al.*, 2020).

Raw data were processed with the software MaxQuant (https://www.maxquant.org/). MS2 spectra were searched by using the Andromeda engine against the FASTA *Quercus*_Database obtained from the translation of *Q. ilex* transcriptome (Guerrero-Sanchez *et al.*, 2017, 2019). Trypsin was set as proteolytic enzyme and a maximum of 2 missed cleavages used in all searches. Precursor mass tolerance was set at 10 ppm, fragment ion mass tolerance at 0.1 Da and charge states at +2 or greater. Peptides were classified into proteins according to the law of parsimony and filtered to a

1% false discovery rate (FDR). Identification confidence was set to a minimum score of 2, proteins with 2 or more peptides matched and at least 15% of sequence coverage. Proteins were quantified in relative terms from the peak areas for precursor ions (the average of the three strongest peptide ion signals) from the identified peptides. Then, they were categorized by function from their FASTA sequences, using the software Mercator v.3.6 (MapMan) (Lohse *et al.*, 2014; http://www.plabipd.de/portal/mercator-sequence-annotation/, accessed January 2021). Uncharacterized proteins were subjected to gene ontology (GO) enrichment (http://pantherdb.org/, accessed March 2021). MS proteomics raw data were deposited with dataset identifier PXD025704 in the ProteomeXchange Consortium via the PRIDE partner repository (Pérez-Riverol *et al.*, 2019).

2.10. Statistical Analysis

The effects of inoculation with *P. cinnamomi* and drought on *Q. ilex* were assessed by using the Kaplan–Meier model, which considers both seedling longevity and status (dead or alive) at the final assessment of survival (Esker al., 2006; Vivas *et al.*, 2012). This model was used in previous studies of tree seedling survival (Solla *et al.*, 2011) and provides an effective tool for identifying survival patterns between treatments where cumulative hazards over time (i.e., hazard functions) are generally proportional. The process involved calculating the area under the Qy curve. Levene's test was previously used to confirm homoscedasticity in the physiological and biochemical variables. Then, the data were subjected to analysis of variance (ANOVA) at $p \leq 0.05$, means being separated with the LSD post hoc test at

$p \leq 0.05$. One-way ANOVA was used to assess the effect of the individual and combined treatments on RLWC; two-way ANOVA to assess the influence of population and treatment as factors in Qy, biochemical parameters and the total content in proteins as measured with the Bradford method; and three-way ANOVA to evaluate the effects of population, treatment and time as factors on physiological parameters as determined by IRGA. Physiological data were previously checked for compliance with the normality and variance homoscedasticity conditions (Sokal and Rohlf, 1995). When the latter were not fulfilled, the Kruskal–Wallis test was used. Some conventional biochemical data were transformed for easier handling. Thus, the phenolic and chlorophyll a contents were used in logarithmic form, starch contents were inverted, and the IRGA parameters (F_v'/F_m') were converted into logarithms for A and squared for Gs and Tr.

The ANOVA analyses were done with the software Statistix v. 10. A random experimental design with drought and inoculation as main factors was used. Significant ($p \leq 0.05$) one-way interactions were subjected to multiple comparisons by least square analysis of means. The significance of pairwise comparisons was determined by using Tukey's test at $\alpha = 0.05$ (Sokal and Rohlf, 1995). The effect of treatments on measured variables was assessed for significance at the 0.05 confidence level. SDS-PAGE band intensities were expressed in relative form by division into the combined intensity of all bands identified in a replicate. Responses were evaluated with provision for repetitiveness between replicates. Significant differences ($p \leq 0.05$) between bands for different treatments and populations were identified by one-way

ANOVA. The raw data from the shotgun analysis were pre-processed for imputation by Random Forests Analysis (https://github.com/Valledor/pRocessomics) and transformed in the same way as in band analysis (i.e., by division into the combined intensity of all bands for each replicate). A Principal Component Analysis (PCA) was performed with RStudio v. 1.3.1093. Changes in protein abundance were assessed on a heat map, using the heatmaply package (Galili *et al.*, 2018) for RStudio v. 4.0.3.

3. Results

3.1 Perlite Water Content, Matric Potential and infection

Perlite water contents on day 32 ranged from 57% in watered seedlings (control and inoculation treatments) to 12% in droughted ones (drought and combined treatments). The previous contents correspond to a matric potential of –15 and –43 kPa, respectively. Seedling infection was evaluated in fine roots from each seedling on days 19 and 32. The presence of *P. cinnamomi* in the roots from the inoculated and combined treatments was identified morphologically (Figure S2).

3.2. Damage symptoms and mortality rate

The effects of the two stresses were examined by inspecting leaf damage visually and recording the number of dead seedlings throughout the experiment. As can be seen from Figure 1, both stresses, but especially the pathogen, reduced plant growth. No chlorosis or wilting was observed in the control seedlings. Leaf damage symptoms included chlorosis, necrosis and

wilting. Damage appeared earlier and was more marked in the presence of both stresses. Thus, damage by effect of the combined stress appeared as early as day 6 in the Al population. Survival at the end of the experiment in the seedlings exposed to *P. cinnamomi* ranged from 50% in inoculated seedlings in the Se population to 0% in seedlings under double stress in the Al population. Based on Kaplan–Meier estimates, survival differed significantly among populations and also among treatments ($p < 0.001$ in both cases; Figure 2). The Al population had the smallest number of living seedlings, followed by the Se and Gr populations. Also, the combined treatment led to the lowest survival, followed by the inoculation, drought and control treatment in this sequence.

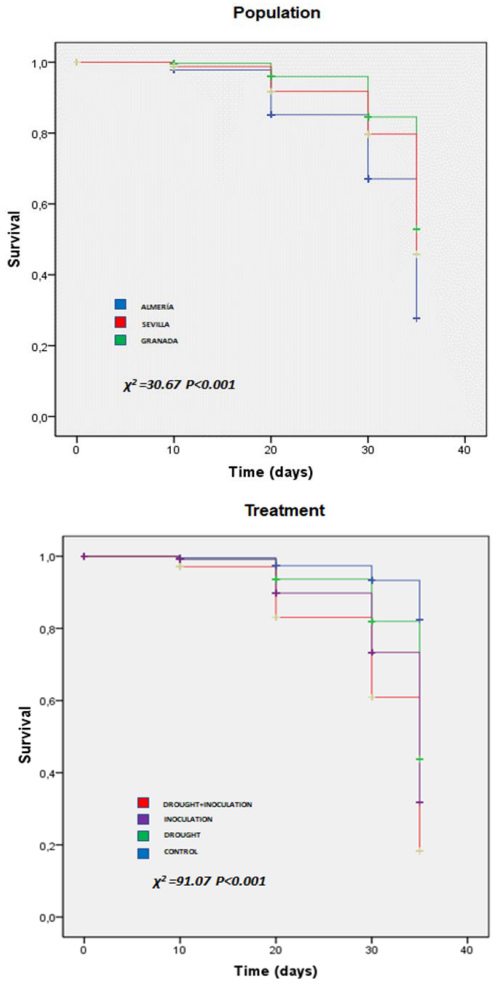

Figure 2. Survival plots for the three *Q. ilex* populations (Almeria, Granada and Seville) under the individual and combined effects of inoculation with *P. cinnamomi* and drought as determined with the Kaplan–Meier model. The units of the x- and y-axis are days and seedling survival rate, respectively.

3.3. Physiological Parameters

Mean relative leaf water contents (RLWC) at the end of the experiment (day 35) ranged from 101.74% with the control treatment in the Se population to 44.22% with the combined treatment in the Al population (Figure S3). No significant differences among populations (p = 0.3037) were observed, however. RLWC was significantly decreased (p < 0.001) by both the inoculation (65–70%) and combined treatments (44–76 %), followed by the drought (75–85%) and control treatments (90–100%) (Figure S3).

The quantum yield of photosystem II (Qy) in the control seedlings as determined in the dark remained nearly constant at 0.60–0.80 throughout the experiment (Figure 3). By contrast, Qy decreased gradually in the seedlings under the drought treatment. The pathogen, both by itself and in combination with drought, resulted in a marked decrease in Qy until day 13, after which the Qy leveled off at ca. 0.19. There were significant differences (p < 0.001) in this respect among treatments, the highest Qy levels corresponding to the inoculation and combined treatments. Also, although no significant differences among populations were observed (p = 0.5032), Al seedlings under the combined treatment behaved differently in this respect from Se and Gr seedlings, with near-zero Qy mean values by day 20.

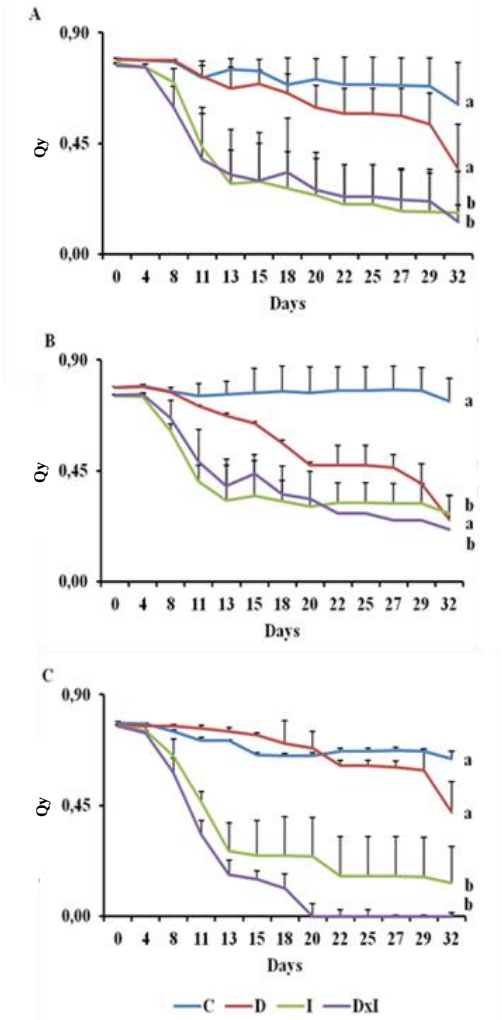

Figure 3. Quantum yield of photosystem II (Qy) in the dark of adapted leaves from the three *Q. ilex* populations (**A** Seville; **B** Granada; **C** Almeria) under the control (C), drought (D), inoculation (I) and combined treatment (D×I). Values are mean ± SE for three biological replicates. Different letters denote significant differences among treatments ($p < 0.05$).

Regarding photosynthetic activity determined on the IRGA analyzer, Fv'/F_m', a measure of photosynthetic efficiency in light-adapted leaves, was reduced by about 63% in the control seedlings from day 8 to day 32 (Figure 4A). There were, however, no statistically significant differences among populations or measurement times ($p = 0.958$). The decrease in F_v'/F_m' was found to significantly depend on the particular treatment ($p < 0.001$), the combined effect being greater than those of the individual stresses. Reductions in the other photosynthetic parameters (A, Gs and Tr) were similar (Figures 4B–4D). Thus, A was decreased from 5.47 to 1.37– μmol CO_2 m^{-2} s^{-1}, Gs from 0.05 to 0.01 mol H_2O m^{-2} s^{-1}, and in Tr from 1.27 to 0.40 mmol H_2O m^{-2} s^{-1} over the period from day 8 to day 32. Again, there were no statistically significant differences among populations or times. The two stresses, both individually and in combination, significantly decreased the previous parameters ($p < 0.001$). The effect, however, was especially marked with inoculation of the pathogen.

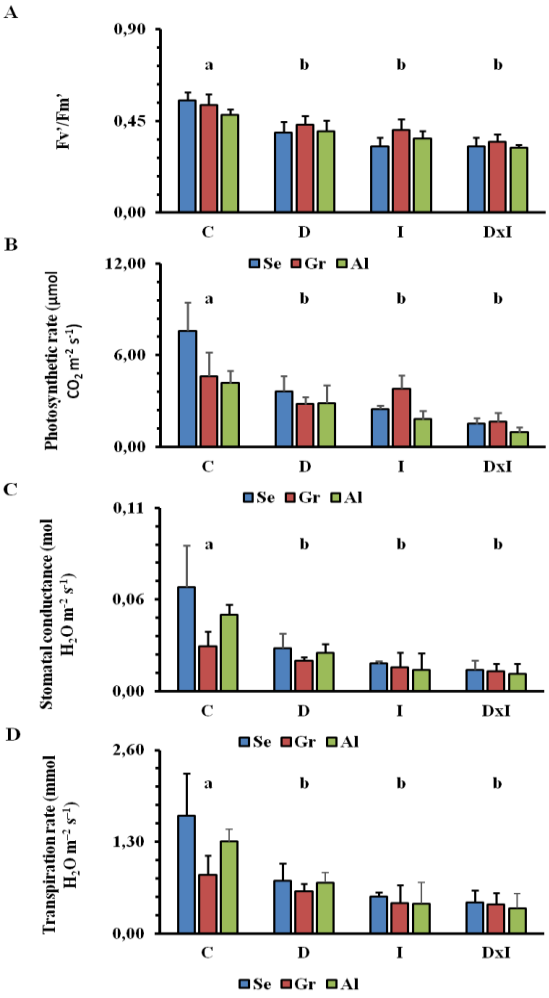

Figure 4. Mean values of Fv′/Fm′ **(A)**, net photosynthesis rate **(B)**, stomatal conductance **(C)** and transpiration rate **(D)** in the Seville (Se), Granada (Gr) and Almeria (Al) populations. Treatments: control (C), drought (D), inoculation (I) and combined (D×I). Different letters denote significant differences among treatments ($p < 0.05$).

3.4. Changes in photosynthetic pigments and metabolites

The results of Figures 2 and 3 led us to subject seedlings from the Se and Al populations, which were the most contrasting among the three, to biochemical and proteomic analyses. The determinations included photosynthetic pigments and metabolites (viz., amino acids, sugars, starch, phenolics and flavonoids) in leaves on day 32. As can be seen from Figure 5, the contents in photosynthetic pigments of the control seedlings in the Se population exceeded those in the Al population. The respective contents in chlorophyll a, in milligrams per gram of fresh weight, were 0.70 ± 0.05 and 0.49 ± 0.02; and those in chlorophyll b 0.39 ± 0.04 and 0.25 ± 0.02. There were no statistically significant differences in chlorophyll a (p = 0.2825) or chlorophyll b contents (p = 0.5036) among treatments. An identical trend was observed in amino acids, with higher contents in the Se population and no significant differences among treatments (p = 0.0686). Sugar contents were higher in the Se population than in the Al population; also, although all treatments increased them, the effect was more marked with inoculation alone than with drought alone or the combined treatment. Conversely, starch was more abundant in the Al population, its highest contents corresponding to the inoculation and combined treatments. By contrast, none of the treatments changed the contents in phenolics, but the combined treatment increased those in flavonoids (especially in the Se population).

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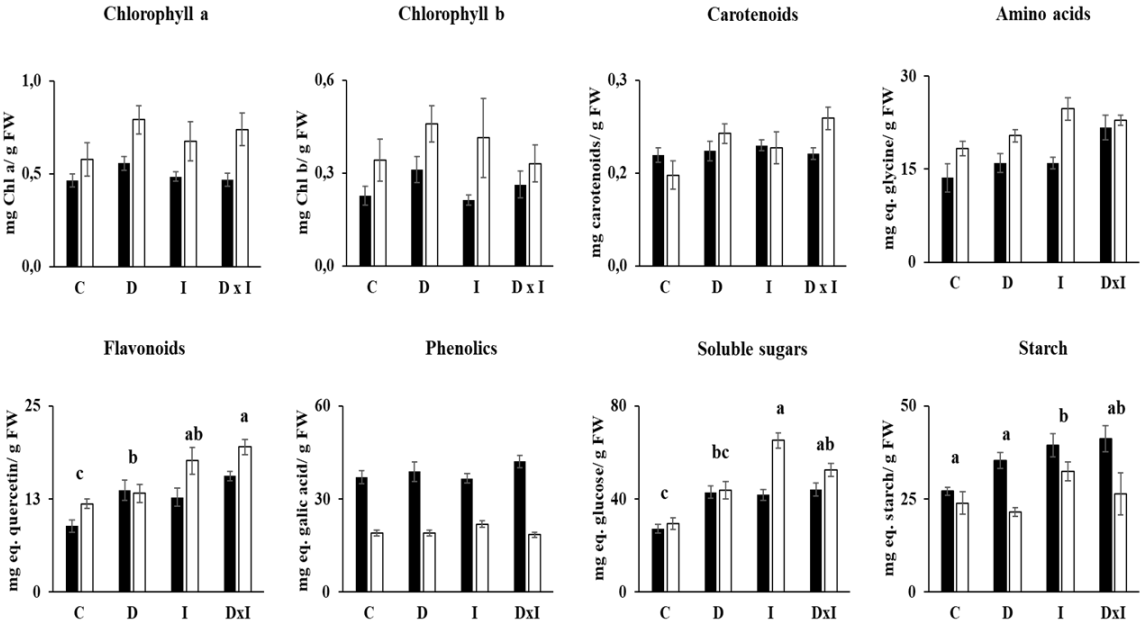

Figure 5. Contents in photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) and metabolites (amino acids, flavonoids, phenolics, soluble sugars and starch) in leaves of *Q. ilex* seedlings from the Seville (white bars) and Almeria populations (black bars). Values are mean ± SE of three biological replicates on day 32. Different letters denote significant differences among treatments ($p < 0.05$).

3.5. Proteomic Analysis

The total amount of proteins extracted with TCA/acetone–phenol and quantified with the Bradford method were similar (0.6–0.9 mg/g FW) in the Se and Al populations irrespective of treatment. 1-D (SDS-PAGE) electrophoresis resolved up to 37 bands that were present in seedlings from both populations whichever the treatment (Figure S4**)**. Twenty-four bands differed statistically among treatments, 9 differing within each population and 6 in both. Most of the resolved bands (11 in total) were more abundant with the combined treatment in at least one population (Table S1). Based on the PCA results, PC1, which explained 40.5% of variability in the Se population, discriminated the control and drought treatments on the one hand, and the inoculation and combined treatment on the other. In the Al population, however, PC1, which explained 37.5% of variability, only discriminated the combined treatment.

Proteins differing between populations and/or treatments were identified by using shotgun analysis, a powerful proteomics platform. The results are summarized in Table 2. Filtering the original dataset (3412 and 2600 positive matches in the Se and Al population, respectively) for confident matches (≥ 2 peptides, ≥ 2 score and ≥ 15% coverage, only those proteins consistently present in the three biological replicates, standard deviation < 50%), provided 414 confidence proteins in the Se population and 734 in the Al population, 318 being shared by the two. Statistical analysis reduced the initial number to 83 in Se and 223 in Al, 25 being shared by the two populations. The whole dataset of shared proteins was categorized in

functional terms by using MERCATOR, which established 17 groups. The best represented groups were carbohydrate metabolism (78 proteins in Se and 116 in Al); folding, sorting and degradation (75 and 141, respectively); energy metabolism (31 and 67); amino acid metabolism (37 and 53); secondary metabolism (29 and 45); ROS scavenging (24 and 18); cellular processes (19 and 31); and defense (12 and 27) (Figure S5).

Table 2. Summary of proteins identified by the shotgun–MS/MS analysis in the two populations and their combination.

	Seville	**Almeria**	**Both populations**
Raw data	3412	2600	2484
Confidence parameters (≥2 Peptides, ≥ 2 Score and ≥ 15% coverage)	2447	2214	2175
Consistent proteins	1110	1214	1015
Standard deviation < 50% between replicates	414	734	318
Statistically significant (*p-value* ≤ 0.05)	83	223	25

Variable proteins fell in 15 groups in Se and 16 in Al, the best represented groups being folding, sorting and degradation (17 and 41, respectively); carbohydrate metabolism (14 and 40); energy metabolism (10 and 15); amino acid metabolism (8 and 23); ROS scavenging (5 and 9); and secondary metabolism (4 and 7) (Figure S6). All functional groups present in the dataset of variable proteins included more up-accumulated proteins —by exception, the energy metabolism group comprised more down-accumulated proteins in

both populations (Figure 6; Figure S6). Based on the number of variable proteins, the proteome was more markedly affected by the treatments in Al than it was in Se (173 vs 45 specific proteins). No treatment-related qualitative differences were observed, however, in any case, the combined treatment induced more proteome changes than did the individual stresses in the Se population; also, the presence of *P. cinnamomi*, both individually and in combination with drought, led to more marked changes in the Al population (Table S1).

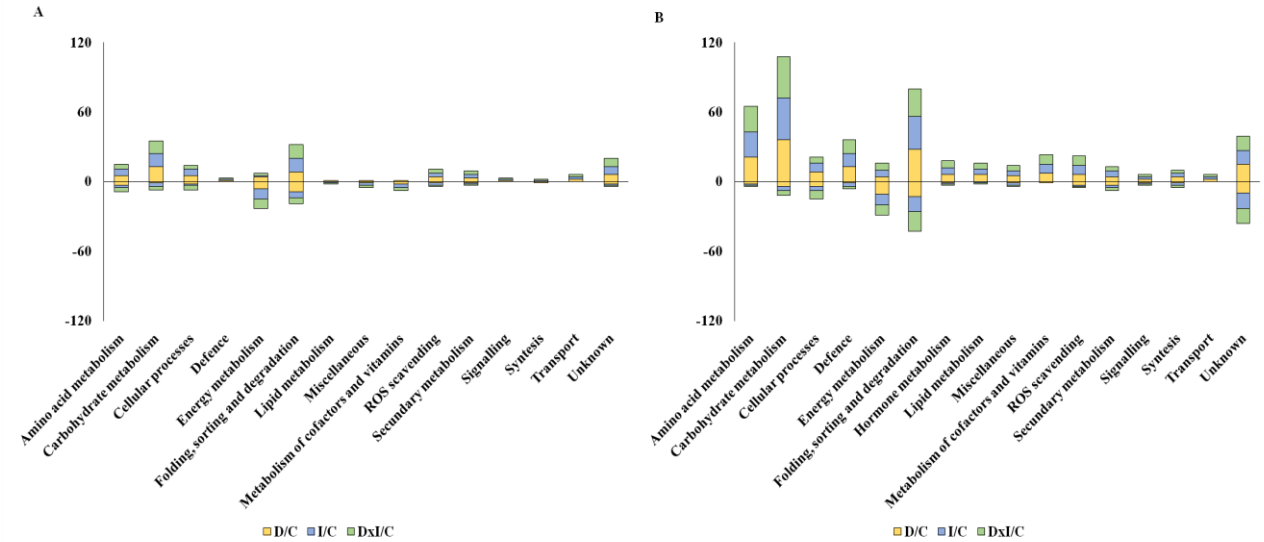

Figure 6. Number of variable proteins up- and downaccumulated whose abundance in the drought (D), inoculated (I) and combined (DxI) treatments was greater or less than with the control treatment (C), in Se **(A)** and Al **(B)** populations. Number of up- and down-accumulated proteins represented as positive and negative values, respectively.

The previous datasets were simplified by PCA. Using the whole set of confidence proteins did not allow separation among populations or treatments with either PC1 or PC2, which accounted for 26% and 15.2% of variability in the data (Figure S7). Differences among treatments in each population were exposed by subjecting the dataset of variable proteins to PCA (Figure 7). In Se, PC1 (46.8% variability) discriminated the control and drought treatments from the inoculation and combined treatments, while PC2 (20.4% variability) discriminated the latter two. In Al, PC1 (51.4%) discriminated the control treatment from all others, and PC2 (17.4%) discriminated drought from the pathogen. The variable proteins most markedly contributing to PC1 and PC2 are listed by functional group in Table 3 and Table S2. In both populations, the greatest positive loadings were those of the carbohydrate metabolism, and folding, sorting and degradation groups.

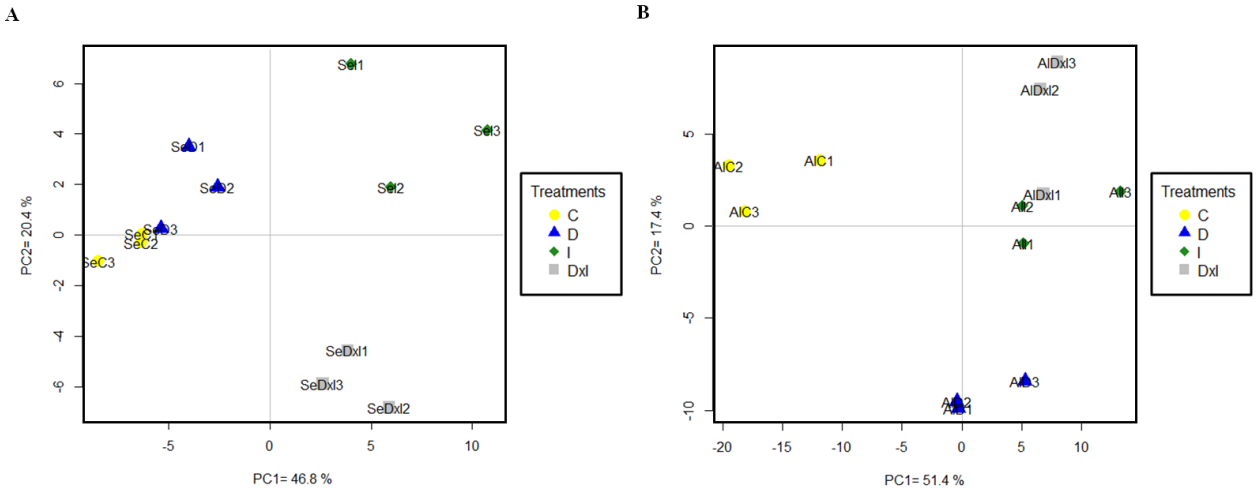

Figure 7. Principal component analysis of variable proteins identified in the Se **(A)** and Al **(B)** populations. Se and Al indicate Seville and Almeria populations. C, D, I and DxI indicate control, droughted, inoculated and combined treatments, respectively. The numbers indicate the biological replicate included in each treatment.

Table 3. List of the first twenty proteins with positive loadings included in PC1 and PC2 of Seville (Se) and Almeria (Al) populations ordered by functional groups. C, D, I and DxI indicate control, drought, inoculated and combined stresses, respectively.

Se population

Functional group	Protein	Up-accumulated	Loadings
PC1			
Amino acid metabolism	Cysteine synthase	I	0.134671201
	Alanine–glyoxylate aminotransferase 2 homologue 1, mitochondrial	I; D×I	0.138725675
Carbohydrate metabolism	6-phosphogluconate dehydrogenase, decarboxylating	I; D×I	0.138255713
	Citrate synthase	I	0.146813054
	Aldehyde dehydrogenase, mitochondrial	I; D×I	0.150395963
Cellular processes	Putative reversibly glycosylatable polypeptide	I	0.145130599
	α-1,4-glucan-protein synthase [UDP-forming] 2	I; D×I	0.148753876
Folding, sorting and degradation	Proline iminopeptidase	I	0.128417337
	Eukaryotic translation initiation factor 3 subunit F	I	0.133480073
	ATP-dependent Clp protease proteolytic subunit-related protein 1, chloroplastic	I	0.138556079
	40S ribosomal protein S5	I	0.139542434

	Translocase of chloroplast	D×I	0.145884352
ROS scavenging	FrnE protein-like	I; C	0.133528064
Secondary metabolism	NADPH-dependent codeinone reductase	D×I	0.141369245
	NADPH:protochlorophyllide oxidoreductase porA	I; D×I	0.142549926
Signalling	Trans-2-enoyl-CoA reductase, mitochondrial	I; D×I	0.130723221
Transport	V-type proton ATPase subunit C	I	0.1433916
Unknown	Outer envelope pore protein 37, chloroplastic	I	0,134589518
	BnaA01g04430D protein	I; D×I	0,138201036
	Carboxylate clamp-tetratricopeptide repeat protein	I; D×I	0,138579354
PC2			
Amino acid metabolism	Glyoxalase I	C; D; I	0.203639693
	Arginine biosynthesis bifunctional protein ArgJ	I	0.204108147
Carbohydrate metabolism	Fructokinase	I	0.120840694
	α-amylase	I	0.134587804
	HMG aldolase	C; D; I	0.191073158
Cellular processes	Cell division protein FtsZ	C	0.121972298
	Patatin	I	0.130841894
	Cell division protein FtsZ homologue 1, chloroplastic	C; D; I	0.153978976

	Harpin binding protein 1	C; D; I	0.215407463
Energy metabolism	Photosystem II subunit P-1	C	0.114099595
Folding, sorting and degradation	Translocase of chloroplast	I	0.136243712
	Mitochondrial processing peptidase	I	0.163693915
	Mitochondrial processing peptidase subunit beta	I	0.170347898
	Peptidyl-prolyl *cis-trans* isomerase	C; D; I	0.173743165
	Clone PI4869 proteasome inhibitor-like protein mRNA	I	0.214512684
Miscellaneous	Oxidoreductase, putative	I	0.200450416
Secondary metabolism	Caffeic acid *O*-methyltransferase	I	0.121922782
Unknown	Cysteine synthase	I	0,119390920
	Fructose-bisphosphate aldolase	I	0,129593872
	Lysine 6-aminotransferase	I	0,173751918

Al population

Functional group	**Protein**	**Up-accumu-lated**	**Loadings**
PC1			
Carbohydrate metabolism	Glucose-6-phosphate 1-dehydrogenase, cytoplasmic isoform 2	D×I	0.083620512
	Aldehyde dehydrogenase	D×I; I; D	0.083884994
	Serine hydroxymethyltransferase	D×I	0.083941959
	Malic enzyme	D×I; I	0.084150059

	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	D×I; I; D	0.084854012
	ATP synthase subunit alpha, mitochondrial	D×I; I; D	0.08562463
	Formate dehydrogenase	D×I; I; D	0.085695392
	Malic enzyme	D×I; I	0.086636987
	Glucose-1-phosphate adenylyltransferase large subunit, chloroplastic/amylo-plastic	I	0.086639036
	Malate dehydrogenase	D×I; I; D	0.088034545
Cellular processes	UDP-D-apiose/UDP-D-xylose synthase	I	0.084723943
	α-1,4-glucan-protein synthase [UDP-forming] 2	D×I; I; D	0.086561347
Energy metabolism	Fructose-bisphosphate aldolase	D×I; I; D	0.086927615
Folding, sorting and degradation	Heat shock protein 60	D×I; I; D	0.085004175
	T-complex protein 1 subunit zeta 1	D×I; I; D	0.089137549
Hormone metabolism	Aluminum-induced protein with YGL and LRDR motifs	D×I; I; D	0.084157903
	Probable aldo–keto reductase 1	D×I; I; D	0.085397904
Lipid metabolism	Acyl-[acyl-carrier-protein] desaturase	I	0.087659507
Miscellaneous	Dynamin-related protein 1E	D×I; I	0.086364345
ROS scavenging	Monothiol glutaredoxin-S17	D×I; I; D	0.083444644
PC2			
Amino acid metabolism	Ornithine carbamoyltransferase, chloroplastic	I; D×I	0.067037801

	Methionine synthase	D×I	0.086406313
Carbohydrate metabolism	Aldehyde dehydrogenase, mitochondrial	D×I	0.069051721
	Glucose-6-phosphate isomerase 1, chloroplastic	D×I	0.089556889
	Serine hydroxymethyltransferase	D×I	0.094407201
	Carbonate dehydratase	D×I	0.105989354
Defense	BURP domain protein RD22	D×I	0.086388976
Energy metabolism	Ferredoxin–NADP reductase	D×I	0.102371216
	Serine–glyoxylate aminotransferase	D×I	0.123546721
Folding, sorting and degradation	40S ribosomal protein S19	D×I	0.068660324
	AMPP	D×I	0.075187168
	50S ribosomal protein L5, chloroplastic	D×I	0.087674819
	60S ribosomal protein L17-2	D×I	0.104318614
Hormone metabolism	12-oxophytodienoate reductase 1	I; D×I	0.068398959
Miscellaneous	CTF2A-like oxidoreductase	D×I	0.098176129
Nucleotide metabolism	Adenylate kinase	I; D×I	0.102221691
ROS scavenging	Catalase	I; D×I	0.109176303
	Catalase	I; D×I	0.118027832
Secondary metabolism	Aminotransferase	I; D×I	0.073624373
Signalling	Sulphite reductase 1 [ferredoxin], chloroplastic	D×I	0.099056469

4. Discussion

In nature, plants are simultaneously exposed to a combination of biotic and abiotic stresses (Ramegowda *et al.*, 2015; Pandey *et al.*, 2017; Teshome *et al.*, 2020). In previous work, our group used molecular methods to investigate the individual effects of *P. cinnamomi* and drought —the two main stresses leading to oak decline (Jorge *et al.*, 2006; Echevarría-Zomeño *et al.*, 2009; Valero-Galván *et al.*, 2013; Sghaier-Hammami *et al.*, 2013; Simova-Stoilova *et al.*, 2015, 2018; San-Eufrasio *et al.*, 2020)— on *Q. ilex* seedlings and their response to these two stresses. In this work, we went one step forward by exploring the combined effects of abiotic (water withholding) and biotic stress (*P. cinnamomi* inoculation) on *Q. ilex* seedlings from three contrasting Andalusian populations (Seville, Granada and Almeria). Only one study of the effects of combined stress on seedling traits and physiology in holm oak had previously been reported which, however, failed to examine metabolic changes or consider the potential influence of plant variability (Ruiz-Gómez *et al.*, 2018). The high inter- and intra-population variability of *Q. ilex* (Valero-Galván *et al.*, 2011; San-Eufrasio *et al.*, 2020), led us to compare several Andalusian populations of this species to gain further insight into the response of holm oak to *P. cinnamomi* and drought.

The effects of stresses and the ensuing response are known to depend on stress intensity and duration. In this work, infection of seedlings was confirmed by isolating the pathogen and identifying it under a light microscope at the end of an experiment where seedlings were placed under

severe drought conditions (see San-Eufrasio *et al.*, 2020).

4.1. Plant mortality and physiological response

The effects of the two stresses and the response of the tolerant phenotype were assessed here through damage symptoms and plant mortality, which differed among populations and treatments. As regards variability in resilience among populations or individuals within populations, the combined effects of *P. cinnamomi* attack and drought are more damaging to survival than are those of the two stressesin isolation (Desprez-Loustau *et al.*, 2006; Sherwood *et al.*, 2015; Ruiz-Gómez *et al.*, 2018; Ghanbary *et al.*, 2017, 2020). A response to attack by *P. cinnamomi* drought stress was first observed in the Al population, which is located in the eastern part of Andalusia. This population is in the farthest region to the place where *P. cinnamomi* root rot was first observed, which was seemingly the southwest of the Iberian Peninsula (Brassier, 1996). This is consistent with the increased susceptibility to *P. cinnamomi* of eastern Andalusian populations previously reported by Sghaier-Hammami *et al.* (2013). On the other hand, *Q. ilex* populations located in the eastern part were previously found to be more tolerant of drought than those in the western part (Valero-Galván *et al.*, 2013; Navarro-Cerrillo *et al.*, 2018), the combination of both stresses having a greater impact on Al than on the other two populations studied here. In turn, the Se and

Gr populations exhibited a similar response to *P. cinnamomi* and drought in terms of damage symptoms and mortality, the earliest symptoms of seedling damage being observed on day 6 in both populations. The Gr population responded more effectively to the individual and combined effects of the two factors; in fact, it was that exhibiting the highest seedling survival at the end of the experiment.

The effects of both stresses and the response of *Q. ilex* to them were also assessed in physiological terms through water status and photosynthetic activity as previously done by Sghaier-Hammami *et al.* (2013) and Valero-Galván *et al.* (2013) in studying individual sources of stress. RLWC was significantly decreased by drought and *P. cinnamomi*, both individually and in combination, relative to the control seedlings, the effect of the pathogen attack being especially marked (De Pascali *et al.*, 2019). However, undamaged *Q. ilex* seedlings maintained their leaf moisture levels, which suggests that they succeeded in holding leaf turgor after 35 days under stressing conditions (Forner *et al.*, 2018). The maximum PSII photochemical efficiency (Qy) and conversion efficiency of PSII open reaction (F_v'/F_m') are commonly used to assess plant response to stress as they are measures of the amount of light energy required for photosynthesis (Bolhàr-Nordenkampf and Öquist, 1993; Filella *et al.*, 1998; Peguero-Piña *et al.*, 2009; Murchie and Lawson, 2013; Sancho-Knapik *et al.*,

2018; Jia *et al.*, 2019). Photosynthetic activity decreased throughout the experiment but differences among populations were not significant (San-Eufrasio *et al.*, 2020). This indicates that the PSII reaction site in *Q. ilex* leaves was affected by the two stresses, which inhibited photosynthesis in the seedlings. Also, the fact the combined treatment led to the lowest *chlorophyll* fluorescence values at the end of the experiment in the Al population is suggestive of an especially synergistic effect of the two stresses on photosynthesis in this population.

An early, fast decrease in net photosynthesis (A), stomatal conductance (Gs) and transpiration rate (Tr) was observed in seedlings under the individual and combined action of *P. cinnamomi* and drought in all populations, which exhibited low values of the three parameters throughout the experiment (Maurel *et al.*, 2001; Sghaier-Hammami *et al.*, 2013; Merilo *et al.*, 2014; Corcobado *et al.*, 2014; Ruiz-Gómez *et al.*, 2018). Early quick stomatal closure in response to *P. cinnamomi* and drought may have reduced water losses and carbon dioxide uptake (Lawlor and Cornic 2002; (Merilo *et al.*, 2014). The quantitative response of these physiological parameters to the individual stresses and their combination was population-independent; in any case, there was a stronger, but not statistically different, response to combined stress (Ruiz-Gómez *et al.*, 2018). Unlike

previously found by Ruiz-Gómez *et al.*, (2018), infection by *P. cinnamomi* had more marked effects than drought, possibly as a consequence of the pathogen damaging the root system and reducing water uptake as a result (Crombie *et al.*, 1990; Corcobado *et al.*, 2013). This reduction in turn may have increased water deficiency, thereby decreasing physiological parameters in the seedlings (Maurel *et al.*, 2001; Robin *et al.*, 2001). Overall, the physiological response of *Q. ilex* to attack by *P. cinnamomi* and drought, whether individually or in combination, was quite similar among populations, the photosynthetic apparatus of the seedlings being affected mainly by the combination of the two stresses.

4.2. Biochemical parameters in undamaged seedlings

The fact that the contents in photosynthetic pigments of undamaged seedlings under stress from drought and *P. cinnamomi* inoculation were similar to those of the control seedlings suggests that the photosynthetic apparatus was altered by neither source of stress (Gallé *et al.*, 2007; San-Eufrasio *et al.*, 2020). Thus, neither amino acids nor phenolics among other biomolecules, were altered in their contents under the stress conditions. The contents in soluble sugars, flavonoids and starch were increased especially markedly in seedlings under both sources of stress, followed by those under drought alone. Abiotic and biotic stresses are known to increase the soluble sugar contents of leaves by regulating expression in genes involved in photosynthesis, osmolyte synthesis and sucrose metabolism (Holland *et al.*,

2016; Khan *et al.*, 2020). Beyond its role as a source of carbon and energy via fermentative or aerobic pathways, soluble sugars promote water uptake to maintain cell volume while avoiding wilting (Manes *et al.*, 2006; Holland *et al.*, 2016). Flavonoids are known to be involved in plant protection responses to pathogens (Treutter, 2006) and have been used to boost drought tolerance by avoiding excessive production of reactive oxygen species (ROS) in plant tissues (Agati and Tattini, 2010). Ghanbary *et al.* (2020) previously found increased flavonoid levels in *Q. infectoria* and *Q. libani* exposed to the combined action of drought and pathogen attack. In this work, we found increased starch accumulation with all treatments relative to the control seedlings, the starch content being especially high in Al seedlings under the inoculation or combined treatment. Starch, phenolic compounds and other defense-related substances were previously found to accumulate in xylem and protoxylem cells in the roots of inoculated plants (Redondo *et al.*, 2015; Ruiz Gómez *et al.*, 2015). This is consistent with the results of Sghaier-Hammami *et al.* (2013), who found increased abundance of proteins involved in starch biosynthesis in response to attack by *P. cinnamomi*. Therefore, although our seedlings responded more strongly to the pathogen than to drought, their metabolism was not imbalanced as a result. In conclusion, the seedlings succeeded in maintaining cellular homeostasis beyond physiological disturbances even under severe stress conditions.

4.3. Alteration of the protein profile by drought and P. cinnamomi

The proteomic techniques used (1D and shotgun-MS/MS analysis) allowed the identification and quantification of a large set of proteins in *Q. ilex*

seedling leaves altered by inoculation with *P. cinnamomi* and/or drought. Based on the number of 1D SDS-PAGE bands and their intensity, the combined treatment had a stronger effect than all others; also, the Al population was more markedly affected than Se population, which is quite consistent with the previous results. Based on the shotgun results, the Al population exhibited more changes in both confidence and variable proteins than did the Se population. This was a consequence of the above-described results as regards damage symptoms and mortality, and suggests that the most susceptible population was that undergoing the greatest proteomic changes. The differences in abundance of proteomic changes were found to dependent on the particular population. Thus, the two populations differed in number of proteins but not in functional categories. The largest groups of confidence and variable up-accumulated proteins in the two populations were those of amino acid metabolism; carbohydrate metabolism; folding, sorting and degradation; ROS scavenging and secondary metabolism (Sghaier-Hammami *et al.*, 2013; Valero-Galván *et al.*, 2013; Hildebrandt, 2018; San-Eufrasio *et al.*, 2021). In contrast, the largest number of down-accumulated proteins in both populations was that of the energy metabolism group (Kapoor *et al.*, 2020), which is in line with the above-described results for physiological parameters. A multivariate analysis revealed a different response to *P. cinnamomi* and drought in the two populations. PCA allowed effective discrimination of the inoculation and combined treatments from the drought and control treatments in the Se population, and also of the control treatment from the individual and combined treatments in the Al population. In addition, PCA clearly discriminated between drought and *P. cinnamomi*

—the latter individually or in combination. Therefore, proteins in both populations were more markedly affected by *P. cinnamomi* attack than they were by drought.

4.4. Putative proteins markers of resilience to combine stress

Those proteins that were consistently found in the three biological replicates from both populations, contributed markedly to variability in the PCA and were more abundant in the combined treatment were taken to be putative molecular markers. The specific proteins considered were aldehyde dehydrogenase, glucose-6-phosphate isomerase, 50S ribosomal protein L5 and alpha-1,4-glucan-protein synthase [UDP-forming]. Aldehyde dehydrogenase levels are known to be raised by both abiotic and biotic stresses (Tola *et al.*, 2021). Thus, plants under stress produce increase amounts of ROS that in turn boost aldehyde production by cells through stress-induced lipid peroxidation (Bartels and Sunkar 2005; Tola *et al.*, 2021). Aldehyde dehydrogenase detoxifies aldehydes by oxidizing them to carboxylic acids (Tola *et al.*, 2021). Sunkar *et al.* (2003) found overexpression of aldehyde dehydrogenase under Arabidopsis conditions to increase tolerance of dehydration. 50S ribosomal protein L5 is a chloroplast ribosomal protein that is upregulated in response to abiotic stress by boosting the synthesis of chloroplast-encoded proteins to offset damage in photosynthesis proteins caused by abiotic stress (Zhu *et al.*, 2021). alpha-1,4-Glucan-protein-synthase, which is involved in the biogenesis or degradation of cell walls, has been identified in response to drought conditions (Fadoul *et al.*, 2018; Dugasa *et al.*, 2021). UDP-forming protein

is associated with the formation of cell walls as physical barriers against pathogens (Shoresh and Harman, 2008). Glucose-6-phosphate isomerase (also called "phosphoglucose isomerase"), a glycolytic enzyme that interconverts glucose-6-phosphate and fructose-6-phosphate, is a drought stress-related protein whose synthesis is boosted under water-deficient conditions (Khanna *et al.*, 2014). This enzyme has been deemed a promoter of starch synthesis by leaves (Backhausen *et al.*, 1997; Yu *et al.*, 2000). Therefore, all the proteins proposed as markers of resilience to combined biotic and abiotic stress —and hence to the decline syndrome in *Q. ilex*— are responsive to adverse environmental conditions, their increased production being a part of the survival mechanisms of this species under restrictive conditions.

5. Conclusions

The presence of stress from *P. cinnamomi* and drought was found to have a synergistic effect on *Q. ilex* seedlings from three contrasting populations in Andalusia, southern Spain. There were no qualitative, but only quantitative differences in the effects and responses to the individual or combined stresses, the Al population being the most markedly affected by the combined treatment —and hence the theoretically most vulnerable to the decline syndrome. As regards individual stresses, drought had a more marked effect than *P. cinnamomi* on the Se and Gr populations, whereas the opposite held for the Al population. Even so, a variable proportion of seedlings from each population responded effectively to stress, with no visible symptoms of leaf damage at any time during the experiment. Those

asymptomatic seedlings responded differently to the individual stresses and their combination. Thus, despite the reduced moisture content and photosynthetic activity, their levels were still high enough for cellular homeostasis to be maintained and differences in the contents of key biomolecules such as photosynthetic pigments, amino acids and phenolics to be insubstantial. The protein functional groups undergoing the greatest changes were folding, sorting and degradation; carbohydrate metabolism; amino acid metabolism; ROS scavenging and secondary metabolism (up-accumulated); and energy metabolism (down-accumulated). The reduction in photosynthetic activity may have arisen from an increase in heterotrophic catabolism. Also, stress-related proteins were more abundant in Al population than they were in the other two. The following proteins are proposed as putative markers of resilience to the decline syndrome in *Q. ilex*: aldehyde dehydrogenase, glucose-6-phosphate isomerase, 50S ribosomal protein L5 and alpha-1,4-glucan-protein synthase [UDP-forming].

6. Supplementary materials

Table S1. List of proteins identified in 1D (SDS-PAGE) and shotgun approaches in control (C), drought (D), inoculated (I), and combined (DxI) treatments from Seville (Se) and Almeria (Al) populations. Different letter indicates that there is significant difference among treatments (*p-value < 0.05)*.

https://drive.google.com/file/d/1s-yJNOIDBDp9hU2RV-hJCy0UiAFFi3-L/view?usp=sharing

Table S2. List of the first twenty proteins with positive and negative loadings included in PC1 and PC2 of Seville (Se) and Almeria (Al) populations ordered by functional groups. C, D, I and DxI indicate control, drought, inoculated and combined stresses, respectively.

Se population

Functional group	Protein	Up-accumulated	Loadings
PC1			
Amino acid metabolism	Cysteine synthase	I	0,134671201
	Alanine--glyoxylate aminotransferase 2 homolog 1, mitochondrial	I; DxI	0,138725675
Carbohydrate metabolism	6-phosphogluconate dehydrogenase, decarboxylating	I; DxI	0,138255713
	Citrate synthase	I	0,146813054
	Aldehyde dehydrogenase, mitochondrial	I; DxI	0,150395963
Cellular processes	Putative reversibly glycosylatable polypeptide	I	0,145130599
	Alpha-1,4-glucan-protein synthase [UDP-forming] 2	I; DxI	0,148753876
Folding, sorting and degradation	Proline iminopeptidase	I	0,128417337
	Eukaryotic translation initiation factor 3 subunit F	I	0,133480073
	ATP-dependent Clp protease proteolytic subunit-related protein 1, chloroplastic	I	0,138556079

	40S ribosomal protein S5	I	0,139542434
	Translocase of chloroplast	DxI	0,145884352
ROS scavending	FrnE protein-like	I; C	0,133528064
Secundary metabolism	NADPH-dependent codeinone reductase	DxI	0,141369245
	NADPH:protochlorophyllide oxidoreductase porA	I; DxI	0,142549926
Signalling	Trans-2-enoyl-CoA reductase, mitochondrial	I; DxI	0,130723221
Transport	V-type proton ATPase subunit C	I	0,143391600
Unknown	Outer envelope pore protein 37, chloroplastic	I	0,134589518
	BnaA01g04430D protein	I; DxI	0,138201036
	Carboxylate clamp-tetratricopeptide repeat protein	I; DxI	0,138579354
Amino acid metabolism	Alpha isopropylmalate synthase	C	-0,122852515
	DS12 from 2D-PAGE of leaf protein, putative	C	-0,118663609
Carbohydrate metabolism	Fructokinase	D	-0,090091392
Cellular processes	Cell division protein FtsZ	C	-0,115483214
Energy metabolism	33kDa oxygen evolving protein of photosystem II	C	-0,134622801
	Post-illumination chlorophyll fluorescence increase protein	C	-0,126020067

	Phosphoglycolate phosphatase 1A, chloroplastic	C; D	-0,125797533
	Sedoheptulose-1,7-bisphosphatase	C; D	-0,119315646
	Ferredoxin--NADP reductase	C	-0,115981200
	Photosystem II subunit P-1	C	-0,111722362
	Putative triosephosphate isomerase	D	-0,107149579
Folding, sorting and degradation	HtrA-like protein	C; D	-0,138145231
	Peptidyl-prolyl cis-trans isomerase	C	-0,116390976
Lipid metabolism	3-oxoacyl-[acyl-carrier-protein] reductase, chloroplastic	C; D	-0,143973693
	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, peroxisomal	C; D; DxI	-0,097117324
Metabolism of cofactors and vitamins	Pyridoxal 5'-phosphate synthase subunit PDX1.3	D	-0,095867000
Miscellaneous	Short-chain dehydrogenase TIC 32, chloroplastic	C	-0,111794987
ROS scavending	Chloroplastic drought-induced stress protein of 32 KDa	C	-0,145548106
Secundary metabolism	Glutamate synthase (Ferredoxin)	C	-0,088592860
Unknown	Metallo-beta-lactamase domain-containing protein	D	-0,101354868
PC2			
Amino acid metabolism	Glyoxalase I	C; D; I	0,203639693
	Arginine biosynthesis bifunctional protein ArgJ	I	0,204108147
Carbohydrate metabolism	Fructokinase	I	0,120840694

	Alpha-amylase	I	0,134587804
	HMG aldolase	C; D; I	0,191073158
Cellular processes	Cell division protein FtsZ	C	0,121972298
	Patatin	I	0,130841894
	Cell division protein FtsZ homolog 1, chloroplastic	C; D; I	0,153978976
	Harpin binding protein 1	C; D; I	0,215407463
Energy metabolism	Photosystem II subunit P-1	C	0,114099595
Folding, sorting and degradation	Translocase of chloroplast	I	0,136243712
	Mitochondrial processing peptidase	I	0,163693915
	Mitochondrial-processing peptidase subunit beta	I	0,170347898
	Peptidyl-prolyl cis-trans isomerase	C; D; I	0,173743165
	Clone PI4869 proteasome inhibitor-like protein mRNA	I	0,214512684
Miscellaneous	Oxidoreductase, putative	I	0,200450416
Secundary metabolism	Caffeic acid O-methyltransferase	I	0,121922782

Unknown	Cysteine synthase	I	0,11939092
	Fructose-bisphosphate aldolase	I	0,129593872
	Lysine 6-aminotransferase	I	0,173751918
Amino acid metabolism	Glutamate decarboxylase	DxI	-0,147065708
	Alanine--glyoxylate aminotransferase 2 homolog 1, mitochondrial	I; DxI	-0,076726628
Carbohydrate metabolism	Glucose-6-phosphate isomerase 1, chloroplastic	DxI	-0,171260663
	ATP-citrate synthase	I; DxI	-0,082207174
	6-phosphogluconate dehydrogenase, decarboxylating	I; DxI	-0,063926424
	Succinate-semialdehyde dehydrogenase	D	-0,042583236
Cellular processes	UDP-glucose 6-dehydrogenase 2	DxI	-0,127661991
	Alpha-1,4-glucan-protein synthase [UDP-forming] 2	I; DxI	-0,039356982
Energy metabolism	Ferredoxin--NADP reductase	C	-0,133181025
	Post-illumination chlorophyll fluorescence increase protein	C	-0,073073096
Folding, sorting and degradation	Haloacid dehalogenase-like hydrolase domain-containing protein At4g39970	DxI	-0,180509786
	50S ribosomal protein L5, chloroplastic	DxI	-0,148120852
	40S ribosomal protein S5	DxI	-0,130680831
Lipid metabolism	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, peroxisomal	C; D; DxI	-0,070512505
ROS scavending	Ascorbate peroxidase	DxI	-0,089730641

	L-ascorbate peroxidase, cytosolic	D; DxI	-0,080329601
	Thylakoid ascorbate peroxidases	I; DxI	-0,066094269
	Chloroplastic drought-induced stress protein of 32 KDa	C	-0,052175967
Secundary metabolism	NADPH-dependent codeinone reductase	DxI	-0,081065206
Transport	Putative beta-subunit of K+ channels	DxI	-0,134098229

Al population

Functional group	**Protein**	**Up-accumulated**	**Loadings**
PC1			
Carbohydrate metabolism	Glucose-6-phosphate 1-dehydrogenase, cytoplasmic isoform 2	DxI	0,083620512
	Aldehyde dehydrogenase	DxI; I; D	0,083884994
	Serine hydroxymethyltransferase	DxI	0,083941959
	Malic enzyme	DxI; I	0,084150059
	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	DxI; I; D	0,084854012
	ATP synthase subunit alpha, mitochondrial	DxI; I; D	0,085624630

	Formate dehydrogenase	DxI; I; D	0,085695392
	Malic enzyme	DxI; I	0,086636987
	Glucose-1-phosphate adenylyltransferase large subunit, chloroplastic/amyloplastic	I	0,086639036
	Malate dehydrogenase	DxI; I; D	0,088034545
Cellular processes	UDP-D-apiose/UDP-D-xylose synthase	I	0,084723943
	Alpha-1,4-glucan-protein synthase [UDP-forming] 2	DxI; I; D	0,086561347
Energy metabolism	Fructose-bisphosphate aldolase	DxI; I; D	0,086927615
Folding, sorting and degradation	Heat shock protein 60	DxI; I; D	0,085004175
	T-complex protein 1 subunit zeta 1	DxI; I; D	0,089137549
Hormone metabolism	Aluminum induced protein with YGL and LRDR motifs	DxI; I; D	0,084157903
	Probable aldo-keto reductase 1	DxI; I; D	0,085397904
Lipid metabolism	Acyl-[acyl-carrier-protein] desaturase	I	0,087659507
Miscellaneous	Dynamin-related protein 1E	DxI; I	0,086364345
ROS scavending	Monothiol glutaredoxin-S17	DxI; I; D	0,083444644
Carbohydrate metabolism	Granule-bound starch synthase 1, chloroplastic/amyloplastic	C	-0,084957955
Cellular processes	Cell division protein FtsZ	C	-0,082806716
	Cell division protein FtsZ	C	-0,078876747
	Chloroplast FtsZ-like protein	C	-0,079957562
Energy metabolism	Photosystem II subunit P-1	C	-0,088594741
	PsbP domain-containing protein 3, chloroplastic	C	-0,083583344

Folding, sorting and degradation	HtrA-like protein	C	-0,087566713
	Probable ADP-ribosylation factor GTPase-activating protein AGD8	C	-0,080434393
	ATP-dependent zinc metalloprotease FTSH, chloroplastic	C	-0,078158888
Miscellaneous	Short-chain dehydrogenase TIC 32, chloroplastic	C	-0,077959055
ROS scavending	Ferredoxin-thioredoxin reductase, variable chain	C	-0,085498846
Secundary metabolism	2OG-Fe(II) oxygenase family oxidoreductase	C	-0,086286533
Syntesis	BnaA04g05670D protein	C	-0,080866612
Unknown	Thylakoid lumenal 19 kDa protein, chloroplastic	C	-0,087292919
	BnaC06g35550D protein	C	-0,086218024
	AT5g48790/K24G6_12	C	-0,08544842
	Thylakoid lumenal protein TL20.3, chloroplastic	C	-0,083354229
	NmrA family protein	C	-0,083036501
	Putative uncharacterized protein Sb01g001480	C	-0,079180102
	BnaAnng11920D protein	C	-0,078428047
PC2			

Amino acid metabolism	Ornithine carbamoyltransferase, chloroplastic	I; DxI	0,067037801
	Methionine synthase	DxI	0,086406313
Carbohydrate metabolism	Aldehyde dehydrogenase, mitochondrial	DxI	0,069051721
	Glucose-6-phosphate isomerase 1, chloroplastic	DxI	0,089556889
	Serine hydroxymethyltransferase	DxI	0,094407201
	Carbonate dehydratase	DxI	0,105989354
Defense	BURP domain protein RD22	DxI	0,086388976
Energy metabolism	Ferredoxin--NADP reductase	DxI	0,102371216
	Serine--glyoxylate aminotransferase	DxI	0,123546721
Folding, sorting and degradation	40S ribosomal protein S19	DxI	0,068660324
	AMPP	DxI	0,075187168
	50S ribosomal protein L5, chloroplastic	DxI	0,087674819
	60S ribosomal protein L17-2	DxI	0,104318614
Hormone metabolism	12-oxophytodienoate reductase 1	I; DxI	0,068398959
Miscellaneous	CTF2A like oxidoreductase	DxI	0,098176129
Nucleotide metabolism	Adenylate kinase	I; DxI	0,102221691
ROS scavending	Catalase	I; DxI	0,109176303
	Catalase	I; DxI	0,118027832
Secundary metabolism	Aminotransferase	I; DxI	0,073624373

Signalling	Sulfite reductase 1 [ferredoxin], chloroplastic	DxI	0,099056469
Amino acid metabolism	Imidazole glycerol phosphate synthase hisHF, chloroplastic	D	-0,122384852
Carbohydrate metabolism	ATP synthase subunit beta, mitochondrial	D	-0,136418545
	Fructokinase	D	-0,126751979
	Enolase	D	-0,120874414
	Bifunctional protein FolD 4, chloroplastic	D	-0,118898426
Cellular processes	Multiple organellar RNA editing factor 2, chloroplastic	D	-0,131707619
	PPIase	D; I	-0,122491011
Defense	DnaK	D	-0,136292566
	Putative chaperone protein ClpB2, chloroplastic	D	-0,132483917
	Heat shock cognate 70 kDa protein	D	-0,124403571
Folding, sorting and degradation	Proteasome subunit beta type-7-A	D	-0,134978520
	Ubiquitin-conjugating enzyme E2 variant 1D	C; D; I	-0,118637713
	Elongation factor Tu, mitochondrial	D; I	-0,112276626

Lipid metabolism	Dihydrolipoyllysine-residue acetyltransferase component 4 of pyruvate dehydrogenase complex, chloroplastic	D	-0,132368462
Folding, sorting and degradation	PSA6	D	-0,140688805
ROS scavending	Ascorbate peroxidase	D	-0,121202464
Signalling	Ran-specific GTPase-activating protein	D	-0,142335340
Transport	Vacuolar H(+)-ATPase	D	-0,113504727
Unknown	Uncharacterized protein At2g27730, mitochondrial	D	-0,131049393
	Major allergen Pru ar, putative	D	-0,128274176

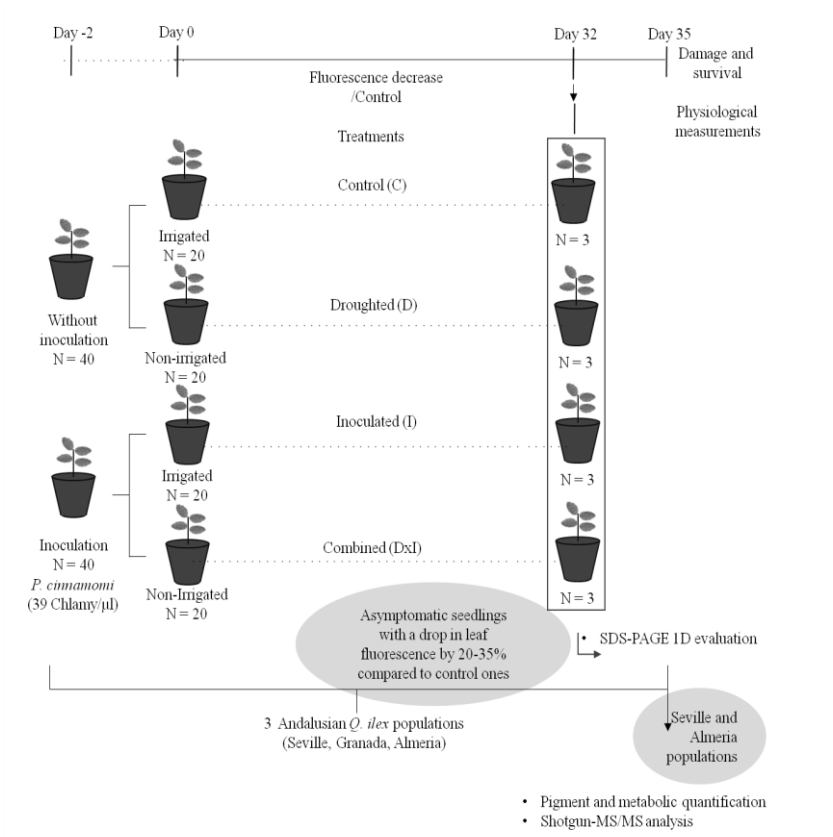

Figure S1. Experimental design used to examine the individual and combined effects of drought and *P. cinnamomi* on *Q. ilex* seedlings. N number of biological replicates. Treatments: C control; D drought; I inoculation; D×I combined.

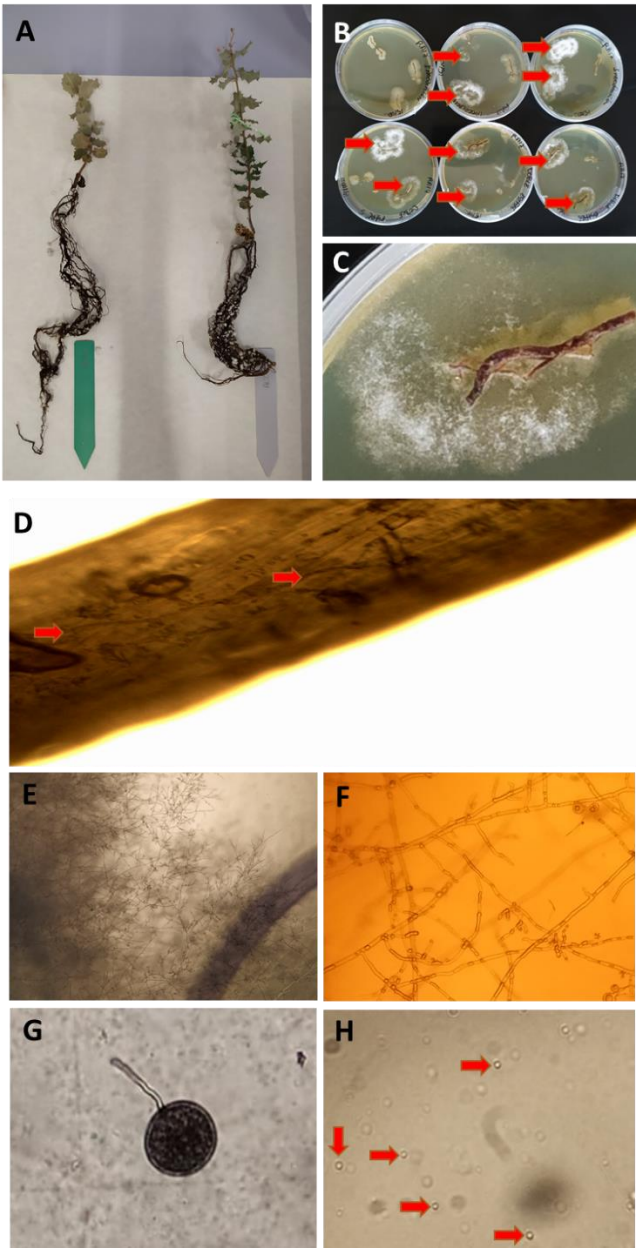
A
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Figure S2. Evaluation of the infection on *Querqus ilex* seedlings inoculated with *Phytophthora cinnamomi*. **(A)** Seedlings with symptoms associated to *P. cinnamomi* infection. Aerial and root damage. **(B)** and **(C)** colonies of *P. cinnamomi* on PARBH medium. **(D)** Hyphae inside the root of *Q. ilex.* **(E)** Mycelium of *P. cinnamomi* (4X magnification). **(F)** Mycelium of *P. cinnamomi* (20X magnification). **(G)** Germinating chlamydospore of *P. cinnamomi*. **(H)** Zoospores of *P. cinnamomi*.

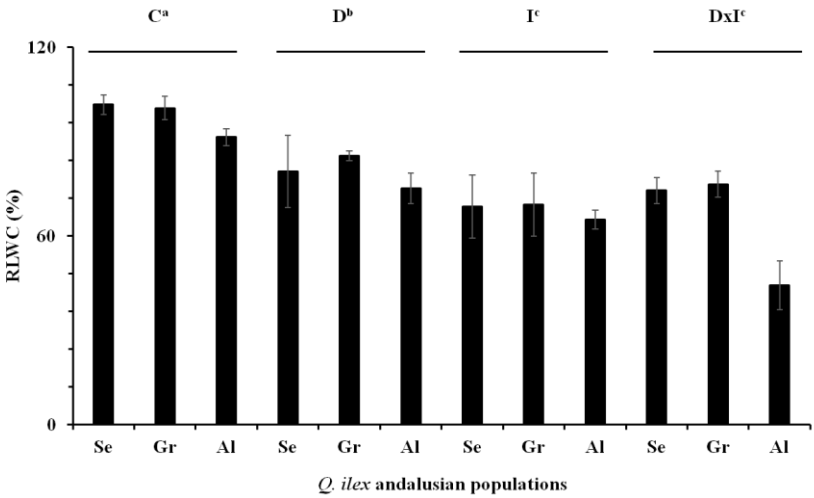

Figure S3. Relative leaf water content (RLWC, %) as determined on day 32 in seedlings from the Seville (Se), Granada (Gr) and Almeria (Al) populations. Values are mean ± SE for three biological replicates. Statistically significant differences among treatments (C control; D drought; I inoculation; I; D×I combined) were observed. Different letters denote significant differences among treatments ($p = 0.0000$).

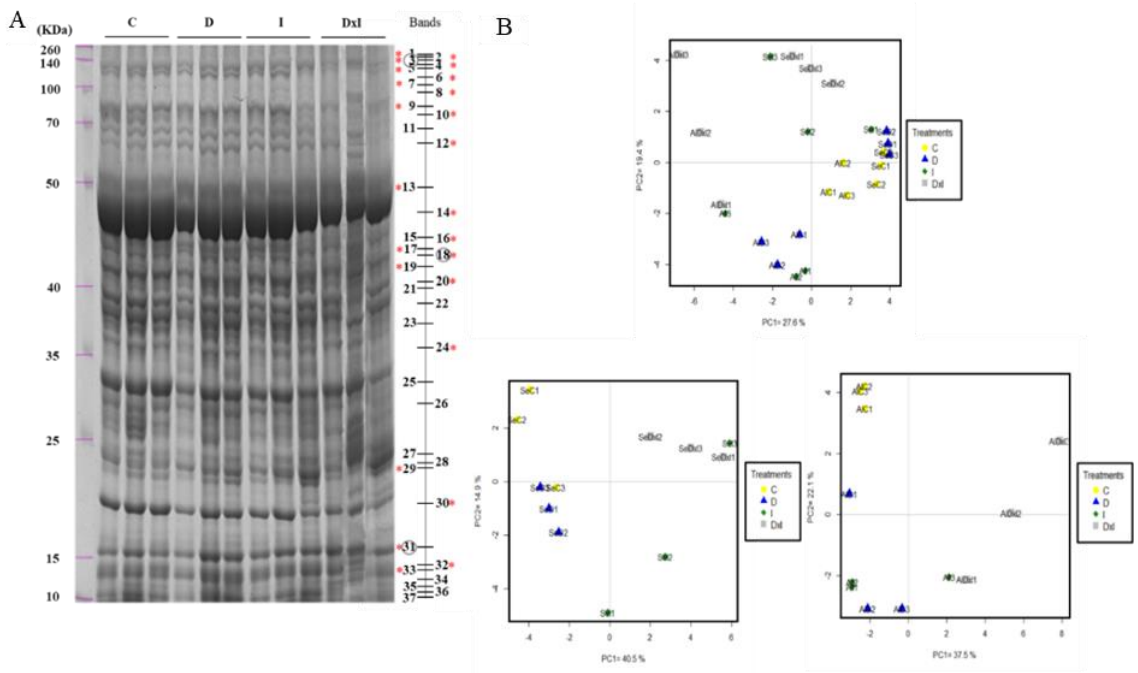

Figure S4. Protein profiles for leaves in seedlings from the Seville (Se) and Almeria (Al) populations as determined by 1-D gel electrophoresis **(A)**. The molecular marker (KDa) is shown on the left of the gel. Principal Component Analysis of all bands identified by 1-D analysis in both Se and Al (top), Se only (bottom-left) and Al only (bottom-right) **(B)**. Treatments: C control; D drought; I inoculation; D×I combined.

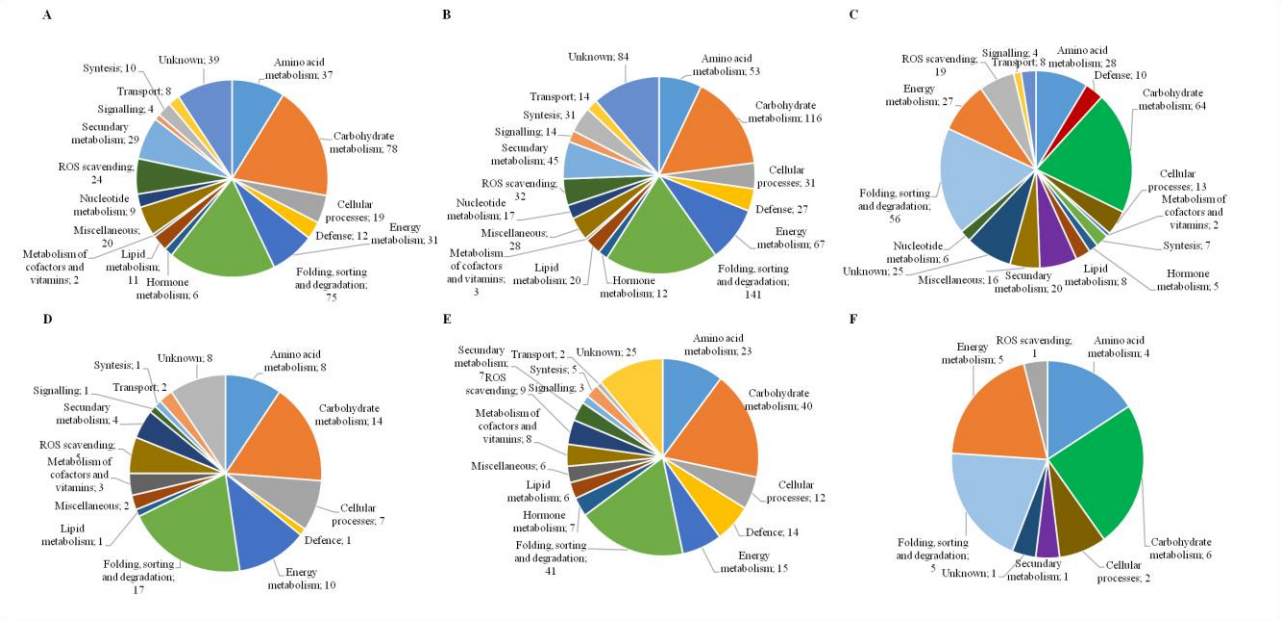

Figure S5. Datasets of total and variable proteins identified in Seville (**A** and **D**, respectively) and Almeria (**B** and **E**, respectively), and shared by the two populations (**C** and **F**, respectively), as grouped by molecular function with MERCATOR. The total number of proteins in each category is shown.

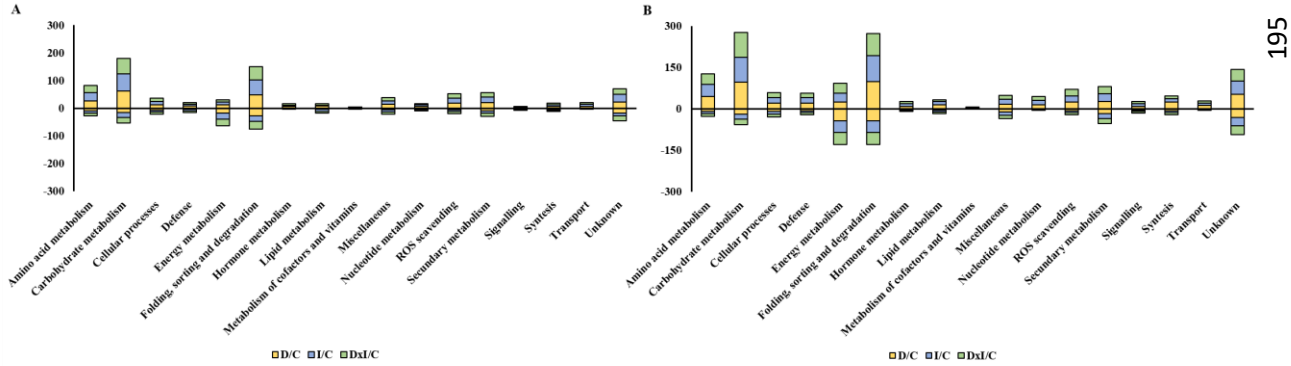

Figure S6. Number of up- and down-accumulated confidence proteins whose abundance in the drought (D), inoculated (I) and combined (D×I) treatments was greater or less than in the control treatment (C) in the Seville **(A)** and Almeria **(B)** populations. The numbers of up- and down-accumulated proteins are represented by positive and negative values, respectively.

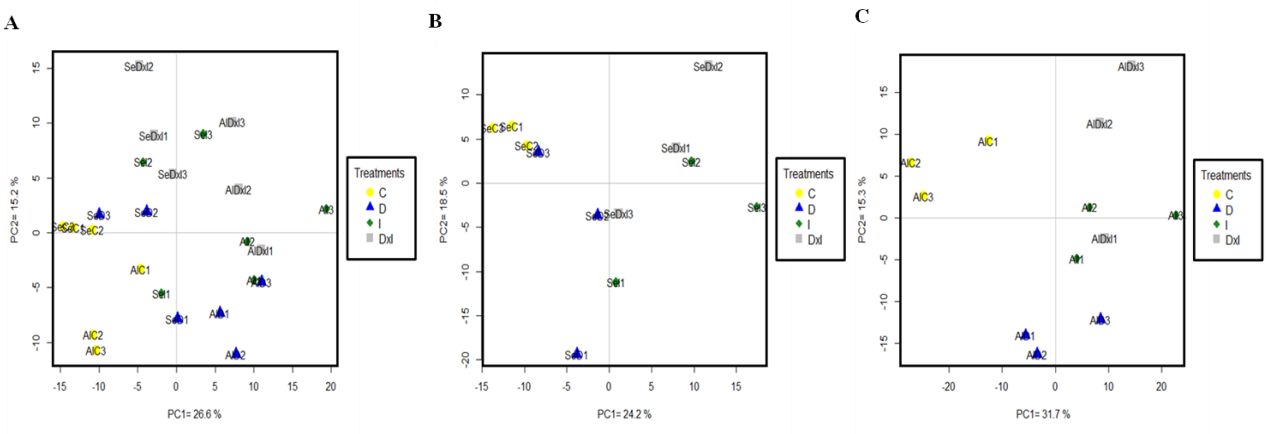

Figure S7. Principal Component Analysis of all confidence proteins identified in both populations (318, **A**), and only in Seville (Se) (414, **B**) or Almeria (Al) (734, **C**).

Chapter V

General discussion

Resilience to adverse environmental conditions (biotic and abiotic stresses) is one of the main objectives in forest tree breeding programs (Matallana-Ramirez *et al.,* 2021). It should be a priority for Holm oak (*Quercus ilex*) taken into account the increasing mortality of individuals associated to the decline syndrome and the predicted conditions in a climate change scenario (Escandón *et al.,* 2021; Sánchez-Cuesta *et al.,* 2021; Gea-Izquierdo *et al.,* 2021). In this direction, the "*Dirección General de Desarrollo Rural, Innovación y Política Forestal del Ministerio de Agricultura, Pesca y Alimentación*" has established the "*National Program for the Conservation and Improvement of the Holm and Cork oaks Genetic Resources against the Decline Syndrome*"

As a non-domesticated species and considering its biological characteristics (long-lived, allogamous, wind-pollinated), the only plausible strategy in breeding programs is the selection of resistant and tolerant elite genotypes for clonal propagation and subsequent use for reforestation programs (Jorrín and Navarro, 2014; Rey *et al.,* 2019; Escandón *et al.,* 2021). In favour of this strategy is the huge phenotypic variability found in the species either within or between populations (Fernández i Marti *et al.,* 2018). Thus, it is commonly observed both symptomatic and asymptomatic individuals close to each other in declined areas (Pérez *et al.,* 2020). Elite or plus genotype trees can be characterized and identified at the morphological, physiological and molecular (nucleic acids, proteins, and metabolites) levels. The research and characterization of the molecular mechanisms and genes implicated in the response and resilience to stresses will favour the selection of elite

genotypes at early stages of the biological cycle, thus speeding breeding programs (Escandón *et al.,* 2021). In this direction, the present Thesis was designed. It aimed at studying the effect and response to individual and/or combined, drought and *P. cinnamomi*, stresses in Holm oak. Both stresses constitute the main factors of the decline syndrome and supposed to be one of the main causes of tree mortality in a climate change scenario (Gea-Izquierdo *et al.,* 2021). It is important to bring to mind that as it occurs in nature, the experimental design consisted in a combination of stresses (Ramírez-Valiente *et al.,* 2019); thus, the drought experiment (Chapter 2 and 3) was performed under high temperature and irradiation, while in the last Chapter 4, the combined effect of drought and *P. cinnamomi* was evaluated. In both cases, severe stress, at the intensity and duration levels, were imposed. The experiment was performed with seedlings considering the high mortality rate observed when transplanting from the nursery to the field, associated mostly to drought conditions (Villar-Salvador *et al.,* 2004; Moreno and Pulido, 2009; Natalini *et al.,* 2016), even thought *Q. ilex* is characterized by its tolerance, and as shown in Chapter 2 and previously reported, much higher than other *Quercus* spp. (Barbeta and Peñuelas, 2016; Gil-Pelegrín *et al.,* 2017; Seleiman *et al.,* 2020). Direct-seeding acorns are more vulnerable to predation and their survival is lower than 1-year-old seedlings (González-Rodríguez *et al.,* 2011). Thus, the use of seedlings in reforestation is highly recommended.

One of the novelties of the works carried out in the thesis was the use of perlite as substrate, which favours a quick and severe stress, facilitating, at

the same time, the collection of tissue samples for molecular experiments in a reasonable experimental time (San-Eufrasio *et al.*, 2020, 2021). To date, a low number of studies have been carried out at molecular level in this genus, in general, and *Q. ilex*, in particular (Escandón *et al.*, 2021). Therefore, an optimal substrate will help to standardize a protocol for future molecular experiments. The studies have been performed at the phenotypic, morphometric, physiologic and molecular levels, being the latter by using classical biochemistry and proteomics. In order to analyse variability at the individual and population levels, seedlings from different Andalusian populations have been employed here.

1. Variability in the resilient phenotypes has been evaluated at different levels:

1.1. Seedling damage symptoms and mortality

Data corresponding to tolerance to drought under high temperature and irradiation have been presented in Chapter 2. Intra- and interpopulation variability has been found with differences between populations based on the number of damaged and dead seedlings throughout the drought experiment (Figure S2). This data showed the existence of geographically close individuals with different phenotypes in terms of abiotic stress tolerance. Thus, the percentage of live and dead individuals within populations should be used as an indicator of the response to drought in this species. This indicator, together with the quality of microhabitats, would be key issues in reforestation programs. Seedlings located under the canopy of oaks in a reforestation stands showed higher seedling growth than tall shrub and open

sites, indicating that sheltered microhabitats are more suitable for oak establishment (Gómez, 2004). Data presented in Chapter 2 (Figure 1) confirm previous observations revealing that eastern populations (Jaen and Granada) showed a lower damage and mortality than western ones (Cadiz, Cordoba, and Seville) (Navarro-Cerrillo *et al.,* 2018). The same conclusions in terms of variability in resilience within and between populations can be deduced from the data corresponding to the combined stress, *P. cinnamomi* and drought (Chapter 4). As expected, the combined effects of the *P. cinnamomi* attack and drought stress were more damaging than the effects of pathogen attack or drought stress alone for the three populations tested (Seville, Granada and Almeria) (Chapter 4, figure 1) (Desprez-Loustau *et al.*, 2006; Sherwood *et al.*, 2015; Ruiz-Gómez *et al.*, 2018; Ghanbary *et al.*, 2017, 2020). The effect of *P. cinnamomi* under drought conditions was firstly observed and was more severe in the Almeria, eastern, population (Chapter 4, figure 2), which is the farthest location of the *P. cinnamomi* entry to Andalusia (Brassier, 1996). This agrees with a higher susceptibility to *P. cinnamomi* in the Andalusian eastern populations previously published by Sghaier-Hammami *et al.* (2013). Although previous studies reported that *Q. ilex* populations located in the eastern part were more tolerant to drought conditions than those located in the western part of Andalusia (Valero-Galván *et al.,* 2013; Navarro-Cerrillo *et al.,* 2018), the combination of both stresses caused a higher effect on Almeria than the other Andalusian populations evaluated in this work. In turn, Seville and Granada populations showed a behaviour pattern similar of response to the *P. cinnamomi* and drought in terms of damage symptoms and mortality, observing the first

damaged seedlings at day 6 in both populations (Chapter 4, figure 2). Granada population showed a better response to the individual and combined stresses, being the population with higher number of seedling survival at the end of the experiment.

1.2. Seedling physiology

Different leaf physiological parameters related to water regime and photosynthesis activity have been measured, including relative leaf water content (RLWC), leaf fluorescence and derived PSII quantum yield at dark-adapted state (Qy), net photosynthesis and stomatal conductance. These parameters have been widely utilized in the study of seedling responses to stresses and taken as an estimation of tolerance and resistance in *Q. ilex* and other *Quercus* species (Peguero-Piña *et al.,* 2009; Martínez-Vilalta *et al.,* 2016; Hosseini *et al.,* 2018; Ghouil *et al.,* 2020).

Although RLWC decreased in response to drought to different degrees depending on the individual and population (the eastern populations showed a higher RLWC that the western ones; Navarro-Cerrillo *et al.,* 2018), *Q. ilex* seedlings kept leaf tissue well hydrated, with values above 43.1 – 70.7% in tolerant or asymptomatic individuals (Chapter 2, Figure 2). This observation is in agreement with previous published data (Valero-Galván *et al.,* 2013; Forner *et al.,* 2018). Similar results were obtained in the combined drought and *P. cinnamomi* experiment included in the thesis, although the reduction of RLWC was more accused in the combined stress (Chapter 4, Figure S3). It is important to remind how *P. cinnamomi*, as previously reported by Sghaier Hammami *et al.* (2013), induced similar responses to that observed

under drought conditions (Manter *et al.*, 2007; Oßwald *et al.*, 2014). The reduction of RLWC in inoculated seedlings was higher than the observed under drought conditions, as previously reported (Forner *et al.*, 2018; De Pascali *et al.*, 2019). However, no significant differences were observed among populations included in the combined experiment (Chapter 4, Figure S3). In conclusion, tolerant individuals kept RLWC and turgor above a threshold value, being one of the key responses to water deprivation stresses. Different mechanisms have been reported in the literature accounting for and efficient use and saving of water resources (Mission *et al.*, 2011; Barbeta and Peñuelas, 2016; Ogaya and Peñuelas, 2021).

Under stress conditions, a reduction in photosynthetic activity was observed as indicated by the decrease in Qy parameters observed in drought, *P. cinnamomi* or combined experiments (Chapter 4, Figure 3). The decrease was dependent on the stress conditions, duration, and population. In agreement with the previous data discussed. The reduction in Qy was maximum in the combined drought and *P. cinnamomi* treatment, and for the Almeria population, indicating a synergy between both drought and *P. cinnamomi* stresses (Chapter 4, Figure 3).

Quercus ilex shows a high sensitivity in stomata conductance to changes in leaf water potential, adjusting it to drought conditions (Gratani *et al.*, 2003). Stomatal closure is a strategy used to diminish water transpiration, prevent leaf water potential decrease, and avoid xylem cavitation under unfavourable conditions (Valladares and Sánchez-Gómez, 2006; Asensio *et al.*, 2007; Gil-Pelegrín *et al.*, 2017). As a consequence, stomatal conductance and net

photosynthesis were diminished. The effect observed in *Q. ilex* was, similarly to other physiological parameters, stress-, duration- and population-dependent (Chapter 2, Figure 4 and Chapter 4, Figure 4). The difference in drought response between western and eastern populations deduced from the previous parameters discussed was not observed while using the stomatal conductance and net photosynthesis parameters. Cadiz population, where most dead seedlings were observed under drought conditions, correlated with the lowest RLWC and the highest stomatal conductance, thus suggesting it to be the least tolerant population amongst the studied ones.

The quantitative response of stomatal conductance and net photosynthesis to combined stresses was independent on population, observing, although not statistically different, a more accused and faster response in the combined stress (Ruiz-Gomez *et al.,* 2018). Regarding individual stresses, unlike described Ruiz-Gomez *et al.* (2018), the infection of *P. cinnamomi* caused lower values than drought conditions, which could be due to damages caused by this pathogen in the root system reducing water uptake (Crombie *et al.,* 1990; Corcobado *et al.,* 2013). This reduction could cause a higher water deficit in the seedlings and thus decreased these physiological parameters at seedling level (Maurel *et al.,* 2001; Robin *et al.,* 2001). So, according to these data, individual or combined stresses caused a reduction in stomatal conductance, and hence a decrease in photosynthesis (Wong *et al.,* 1979; Flexas and Medrano, 2002). This should cause a reduction in the supply of ATP and NADPH, and the activation of heterotrophic catabolic pathways

using starch and other biomolecules as fuels. At the same time, a reduction in photosynthesis prevents the formation and accumulation of toxic reactive oxygen species (Salehi-Lisar and Bakhshayeshan-Agdam, 2016).

1.3. Seedling biochemical response

The third level of study included the analysis of different biomolecules by using classical colorimetric biochemical techniques (López-Hidalgo *et al.,* 2021). Thus, chlorophylls, carotenoids, amino acids, starch, sugars, phenolics and flavonoids were quantified in leaf tissue from control and stressed, non-damaged, seedlings at the end of the experiment (25 and 32 days for the drought and combined experiments). In general, photosynthetic pigments remained unchanged under stressing conditions, being in the range of those corresponding to control seedlings. However, significant differences were observed at population level (Chapters 2, Figure 5 and Chapter 4, Figure 5). This observation could be species-, genotype- and/or experiment-dependent, as in a previous study, a decrease in chlorophyll and an increase in carotenoids was reported for *Q. robur, Q. coccifera, and Q. ilex* (Spyropoulos and Mavrommatis, 1978). The differences among populations could reflect differences in photosynthesis activity.

An increase in the content of sugar, amino acids and total phenolics was observed under drought conditions, which is expected in those tolerant species prone to drought (Rivas-Ubach *et al.*, 2014). In the *P. cinnamomi* or combined stress experiment, neither amino acid nor phenolic content was modified, indicating that the biochemical response was stress-dependent. On the contrary, sugar, flavonoids and starch were increased in inoculated and

combined stress experiments. Sugars, phenolics and amino acids, contribute to plant responses to biotic and abiotic stresses and to the seedling survival, preventing water loss and enhancing osmoprotection as well as preventing oxidative damage (Rivas-Ubach *et al.,* 2014). Abiotic and biotic stresses increase the soluble sugar content in leaf, regulating gene expression involved in photosynthesis, osmolyte synthesis and sucrose metabolism (Holland *et al.,* 2016; Khan *et al.,* 2020). Beyond its role as a source of carbon and energy via fermentative or aerobic pathways, soluble sugars promote water uptake to maintain cell volume avoiding wilting (Manes *et al.,* 2006; Holland *et al.,* 2016). The increase in the sugar content could be explained by the mobilization of starch (Rodriguez-Calcerrada *et al.,* 2018; Simova-Stoilova *et al.,* 2018). Previous studies showed that starch reserves were depleted in the conversion to soluble sugars during drought (Maguire and Kobe, 2015). Amino acids, such as proline and glycine have been described as active osmotic compounds, and their increase in response to drought has been previously reported in *Q. ilex* (Rodriguez-Calcerrada *et al.,* 2018). Several phenolic compounds with an antioxidant function have been described in *Q. ilex*, such as gallic acid, isoloquiritigenin or catechin (López-Hidalgo *et al.,* 2018). Our study found a significant increase in the content of total phenolics in the non-irrigated seedlings as previously reported (Chapter 2, Figure 5) (Rivas-Ubach *et al.,* 2014), and that of flavonoids in inoculated seedlings. This induction could be directly related to a direct response to scavenge the increase in the levels of reactive oxygen species caused by the stress. However, anthocyanins displayed a significant decrease under drought conditions that was contrary to previous published results

(Spyropoulos and Mavrommatis, 1978), although a direct relationship between anthocyanin accumulation and drought tolerance does not always happen (Hughes *et al.*, 2010). Flavonoids are known to be implicated in plant protection against pathogens (Treutter, 2006). As happened in *Q. infectoria* and *Q. libani*, an increased in flavonoids was observed when exposed to the combined impacts of drought and pathogen attack (Ghanbary *et al.*, 2020).

The data of starch content is apparently contradictory according to the hypothesis previously discussed in which it is expected to be mobilized and reduced under stressing conditions. Thus, an increase in the starch content has been observed in Almeria population in inoculated or combined stress treatments (Chapter 4, Figure 5). This agrees with data published by Sghaier-Hammami *et al.* (2013) where an increase in the abundance of proteins related to starch biosynthesis was described in response to *P. cinnamomi*.

In conclusion, no changes at photosynthesis pigment level seem to indicate little or no damage to the photosynthesis molecular machinery under drought and/or *P. cinnamomi* (Epron *et al.*, 1992; Gallé *et al.*, 2007). Also, it suggests that there was a correct function of the metabolism and metabolic homeostasis, with changes in the metabolic pattern, as above indicated from autotrophic to heterotrophic, and inducing the synthesis of stress-related metabolites acting as osmolytes (sugars and amino acids) and protecting against cellular damage caused by oxygen reactive species (phenolics).

1.4. Seedling proteomics response

The fourth level of study was the analysis of the changes in the leaf protein profile in response to individual or combined stresses by using a proteomics approach. The proteomics analysis had a double objective, the first one related to the identification of mechanisms and gene products related to the stress-resilient character and the second one focussed on the proposal of protein markers to be used in the identification of elite genotypes. For that, a triple strategy was used: gel-based, gel-free or shotgun proteomics (Chapter 4), and targeted post-acquisition data analysis (Chapter 3). In all the cases proteins were identified by using a species-specific *Q. ilex* transcriptome database constructed in our research group (Guerrero-Sánchez *et al.,* 2017, 2019, 2021). To date, many works have been performed in the group to explore the response to individual stress, either *P. cinnamomi* (Sghaier-Hammami *et al.,* 2013) or drought (Jorge *et al.,* 2006; Echevarría-Zomeño *et al.,* 2009; Valero-Galván *et al.,* 2013; Sghaier-Hammami *et al.,* 2013; Simova-Stoilova *et al.,* 2015, 2018) from a proteomics point of view. In the drought experiments, the proteomics study was performed with just four populations, (Cadiz, Granada, Huelva and Seville), that were selected based on data presented in chapter 2, and were representative of the Andalusian eastern and western parts, with differences levels of tolerance to drought. The proteomics data have been deeply discussed in Chapter 3, and because of that the most relevant aspects, related to previous physiological data and the panel of protein and peptide markers, will be mentioned in this general discussion.

Despite a clear reduction in photosynthetic activity under drought conditions, the molecular photosynthetic machinery was seemingly unaffected; in fact, only a few photosynthesis proteins exhibited any changes, and only one (chlorophyll a-b binding protein) was included in the marker panel. This result is consistent with data in photosynthetic pigments that were not altered under drought stress.

Starch degradation in response to stress has been often associated with improved tolerance and potentially limited photosynthesis (Cuellar-Ortiz *et al.*, 2008; González-Criz and Pastenes, 2012). Sugars resulting from starch degradation, and other derivative metabolites, help plants grow under stress and function as osmoprotectants and compatible solutes to mitigate the adverse effects of stress (Krasensky and Jonak *et al.*, 2012) as found in droughted *Q. robur* (Sergeant *et al.*, 2011). Although environmental factors are known to have strong effects on the starch synthesis, their regulatory mechanisms remain unclear (Thalmann and Santelia, 2017). Some studies reported increased starch accumulation under stress, mainly in response to high salinity or cold (Kaplan and Guy, 2005; Yin *et al.*, 2010; Skirycz *et al.*, 2010). In this work, two proteins of carbohydrate metabolism of the marker panel (viz., granule-bound starch synthase 1, which is chloroplastic/ amyloplastic, and the glycosyl hydrolase family protein with chitinase insertion domain) were found at increased levels in, mainly, the Seville population. However, several starch degradation proteins not selected as putative markers were significantly increased in response to drought in some of the experimental conditions studied, including phosphoglucan water

dikinase, alpha-glucan phosphorylase and alpha-amylase. Although apparently contradictory, this response may be related to the presence of different types of starch, whether permanent or transitory and that of isoforms involved in their synthesis and mobilization (Kaplan and Guy, 2005; Wang *et al.,* 2006; Prathap and Tyagi., 2020).

The broadest group of proteins and derived peptides selected as putative markers of drought tolerance consisted of redox (2-alkenal reductase NADP-dependent, short-chain alcohol dehydrogenase A, disulfide-isomerase) and stress response proteins (endoplasmin, dehydrin, senescence/dehydration-associated protein and aldo-keto reductase). Some were closely associated with drought in several studies on the genus *Quercus* (Echevarria-Zomeño *et al.,* 2009; Sergeant *et al.,* 2011) or with biotic stress caused by *P. cinnamomi* (Sghaier-Hammami *et al.,* 2013). Furthermore, a representative number of redox proteins not included in the marker panel have been identified as being increased to a greater or lesser extent in response to drought, including glutathione S-transferase, glutathione peroxidase, thioredoxin, peroxidase, superoxide dismutase, lipoxygenase, among others. The marker panel also included two enzymes involved in the shikimate–phenolic biosynthetic pathways, namely: chalcone synthase and 3-phosphoshikimate 1-carboxyvinyltransferase, which is in good agreement with the drought-induced increase in phenolic content observed and data reported in the literature for *Q. ilex* (Nogues *et al.,* 2013; San-Eufrasio *et al.,* 2020) and other *Quercus* spp. (Jafarnia *et al.,* 2018; Ghanbary *et al.,* 2020).

Changes in synthesis and degradation of proteins under stress conditions,

such as drought, can be interpreted as a mechanism of adaptation through the installation of the translational apparatus and protein synthesis by recycling available amino acids in plants through protein degradation. Thus, plants respond to drought by synthesizing protective proteins and repairing or degrading damaged ones (Vaseva *et al.,* 2012). Considering the general changes observed in the proteome in response to drought, synthesis (ribosomal and transcription) was the most represented group of proteins showing qualitative and quantitative changes, followed by folding and degradation category. Many of the proteins selected as putative markers here are involved in synthesis processes; such is the case, for instance, with translation initiation factor, zinc finger protein VAR3, RNA-binding protein, and methionyl-tRNA synthetase. The marker panel also included folding and degradation proteins, such as the chaperones calreticulin and GrpE protein, and serine protease subtilisin. 2DE-MSMS proteomic analysis previously revealed a similar response involving some of the previous proteins in *Q. ilex* (Valero-Galvan *et al.,* 2013; Simova-Stoilova *et al.,* 2015) and *Q. robur* (Sergeant *et al.,* 2011) under drought and suggested active metabolic adjustment to stress.

Transport proteins, such as the water channel protein aquaporins, have been associated with plant tolerance of biotic and abiotic stresses, to which they respond by regulating the movement of water and small molecules through plasma membranes and vacuoles (Li *et al.,* 2015). Based on a proteomics strategy involving the identification of proteotypic peptides, some transport proteins have been proposed as markers of tolerance to drought (Castillejo *et*

al., 2016) and resistance to *Ascochyta* blight (Castillejo *et al.*, 2020) in pea. The proteins were assumed to induce signalling and transport processes as mechanisms to maintain homeostatic equilibrium and cope with stress. The proposed putative markers in this study included the importin subunit alpha, aquaporin and mitochondrial fission 1 protein A, although other transport and signalling proteins, such as 14-3-3-like protein, lipocalin, outer envelope pore protein, voltage dependent anion-selective channel and translocase of chloroplast 90 protein, were also more represented under drought in some of the experimental conditions in this study.

At the proteomics level, the two populations analyzed (Almeria and Seville) in the combined stress experiment behaved differently, as revealed by gel-based and gel-free approaches, and differed from the observed under drought and *P. cinnamomi* infection conditions, revealing, once more, a response genotype- and stress-specific. Differences in the type of stress were quantitative more than qualitative, with no stress-specific variable proteins found. Thus, Almeria population was more affected than Seville one, with, respectively, 83 and 223 variable proteins respectively, being only 25 common to both. However, when analyzing the functional groups of variable proteins, the same tendency was found, in which an accumulation of stress- and defense-related proteins, antioxidant and phenolic pathway enzymes, and a reduction of those of photosynthesis was observed. The panel of consistent up-accumulated proteins in the two populations tested in this combined stress experiment resulted in 4 proteins, which are proposed as putative molecular markers (Chapter 4, Table 3). It included redox enzymes

such as aldehyde dehydrogenase, glycolytic enzymes such as glucose-6-phosphate isomerase, and those involved in cell wall biogenesis, such as alpha-1,4-glucan-protein-synthase, and the protein of folding 50S ribosomal L5.

An increase of aldehyde dehydrogenase was observed in the abiotic and biotic stresses (Tola *et al.,* 2021). When plants are subjected to stress, there is an increase in the formation of ROS that causes an increase of aldehydes in cells as the result of stress-induced lipid peroxidation (Bartels and Sunkar 2005; Tola *et al.,* 2021). Aldehyde dehydrogenase is responsible for efficient detoxification of aldehydes by oxidizing them to carboxylic acids (Tola *et al.,* 2021). In Arabidopsis, the overexpression of aldehyde dehydrogenase increased dehydration tolerance (Sunkar *et al.,* 2003). 50S ribosomal protein L5 is a chloroplast ribosomal protein that is upregulated in response to abiotic stress, promoting the synthesis of chloroplast-encoded proteins to compensate the damaged photosynthesis proteins caused by an abiotic stress (Zhu *et al.,* 2021). Alpha-1,4-glucan-protein-synthase has been identified in response to drought conditions, being related to the biogenesis or degradation of cell wall (Fadoul *et al.,* 2018; Dugasa *et al.,* 2021). UDP-forming is associated with the formation of cell wall involved in physical barriers in the attack of a pathogen (Shoresh and Harman, 2008). Glucose-6-phosphate isomerase, also known as phosphoglucose isomerase is a glycolytic enzyme that interconverts glucose-6-phosphate and fructose-6-phosphate. It has been described as a drought stress-related protein that is more abundant in response to water-deficit stress (Khanna *et al.,* 2014). This enzyme has been

described as a promoter of synthesis of starch in leaves (Backhausen *et al.*, 1997; Yu *et al.*, 2000).

So, in summary, all the proteins proposed as markers of resilience to combined biotic and abiotic stress, and hence, to the *Q. ilex* decline syndrome have been reported as responsive to adverse environmental conditions, being its increase a part of the mechanisms responsible of the survival under restrictive conditions. However, and considering the high variability found in *Q. ilex*, the present study has limitations considering that just a few individuals from different populations have been analyzed deeply. So, the proposed mechanisms of tolerance/resistance and putative protein markers should be validated to be considered as general for the species. However, attending to the protein profile pattern on the populations studied we could speculate on similar responses to the studied stresses perhaps due to geographic proximity. Thus, based on the proteomics results the differences found between the populations studied may have a geographical explanation as has already been described by Fernandez i Marti *et al.* (2018), suggesting that the Guadalquivir Valley has played an important role in determining population divergence.

Conclusions

1. Among the *Quercus* species surveyed (*Q. robur, Q. faginea, Q. pyrenaica, Q. suber,* and *Q. ilex*), *Q. ilex* was the most drought tolerant. Within *Q. ilex* intra- and interpopulation variability has been found, with the Andalusian Eastern populations (Jaen and Granada) being more drought tolerant than the Western ones (Cadiz, Cordoba and Seville).

2. The combined *P. cinnamomi* and drought effect on the three populations tested (Seville, Granada and Almeria) was more damaging than that of pathogen inoculation or drought stress alone, being the Almeria and Granada populations the most and least resilient to the decline syndrome respectively.

3. Leaf relative water content decreased in response to drought and/or *P. cinnamomi* to different degrees depending on the individual, population, and treatment, being more intense in the combined stress. Tolerant individuals kept leaf tissue well hydrated, with values of relative water content above 40%.

4. The decrease observed in the physiological parameters (Qy, net photosynthesis and stomatal conductance) was dependent on the stress conditions, duration, and population, being maximum in the combined drought and *P. cinnamomi* treatments, and in Almeria population.

5. Photosynthetic pigments remained unchanged under stressing conditions, revealing integrity of the photosynthetic molecular machinery.

6. Metabolic homeostasis and reorganization of the pathways, from autotrophic to heterotrophic, and the increase of stress related ones (antioxidants, osmotic active compounds) was a general response to the stress treatments; with particularities for biotic and abiotic stresses, and with no qualitative, but quantitative differences among populations and individuals.

7. Changes in the leaf protein profiles was stress- and individual-dependent, with general tendencies for all the treatments and populations. Variable proteins belonged to different functional groups to photosynthesis, sugar metabolism, stress-related and transport. While photosynthesis proteins were little affected (drought) or decreased (combined stress), those of sugar and phenolic metabolism, antioxidant redox enzymes, and stress-related were increased under abiotic and biotic stresses.

8. A panel of 30 proteins and their 46 derived peptides were proposed as putative markers of drought tolerance. Two of them, subtilisin and chaperone GrpE, were found at increased levels in three out of four populations surveyed. Regarding the total variable proteins found in the combined stress experiment, four were proposed as putative markers of resilience, including an aldehyde dehydrogenase, glucose-6-phosphate isomerase, 50S ribosomal protein L5, and alpha-1,4-glucan-protein synthase [UDP-forming]. These six proteins were proposed as putative markers of resilience, making them especially interesting for validation in subsequent experiments. They should be confirmed for a higher number of populations and individuals before being considered as general for *Q. ilex* species.

Conclusiones

1. Entre las especies de *Quercus* estudiadas (*Q. robur*, *Q. faginea*, *Q. pyrenaica*, *Q. suber* y *Q. ilex*), *Q. ilex* fue la más tolerante a la sequía. Dentro de esta, se ha encontrado variabilidad intra e interpoblacional, siendo las poblaciones orientales andaluzas (Jaén y Granada) más tolerantes a la sequía que las occidentales (Cádiz, Córdoba y Sevilla).

2. El efecto combinado de *P. cinnamomi* y sequía sobre las tres poblaciones ensayadas (Sevilla, Granada y Almería) fue más intenso que el estrés por separado de inoculación con el patógeno o el estrés por sequía, siendo las poblaciones de Almería y Granada las más y menos resistentes al síndrome de la seca, respectivamente.

3. El contenido hídrico relativo de las hojas disminuyó en respuesta a la sequía y/o a *P. cinnamomi* en distinto grado según el individuo, la población y el tratamiento, siendo más acusado en el estrés combinado. Los individuos tolerantes mantuvieron el tejido foliar bien hidratado, con valores de contenido hídrico relativo superiores al 40%.

4. La disminución observada en los parámetros fisiológicos (Qy, fotosíntesis neta y conductancia estomática) fue dependiente de las condiciones de estrés, duración y población, siendo máxima en los tratamientos combinados de sequía y *P. cinnamomi*, y en la población de Almería.

5. Los pigmentos fotosintéticos se mantuvieron sin cambios bajo las condiciones de estrés, revelando la integridad de la maquinaria fotosintética.

6. La homeostasis metabólica y la reorganización de las vías, de autotróficas a heterotróficas, y el aumento de las relacionadas con el estrés (antioxidantes, compuestos osmóticamente activos) fue una respuesta general a los tratamientos de estrés; con particularidades para los estreses bióticos y abióticos, y sin diferencias cualitativas, pero sí cuantitativas, entre poblaciones e individuos.

7. Los cambios en el perfil proteico de las hojas fueron dependientes del estrés y de los individuos, con tendencias generales para todos los tratamientos y poblaciones. Las proteínas variables pertenecían a diferentes grupos funcionales relacionados con la fotosíntesis, el metabolismo de azúcares, relacionados con el estrés y de transporte. Mientras que las proteínas de fotosíntesis se vieron poco afectadas (sequía) o disminuyeron (estrés combinado), las del metabolismo de azúcares y fenólicos, enzimas antioxidantes y las relacionadas con el estrés aumentaron bajo estrés abiótico y biótico.

8. Se propuso un panel de 30 proteínas y 46 péptidos derivados como marcadores putativos de tolerancia a la sequía. Dos de ellos, la subtilisina y la chaperona GrpE, se encontraron incrementadas en tres de las cuatro poblaciones estudiadas. En cuanto a las proteínas variables totales encontradas en el experimento de estrés combinado, cuatro se propusieron como marcadores putativos de resistencia, incluyendo aldehído deshidrogenasa, glucosa-6-fosfato isomerasa, proteína ribosomal 50S L5 y alfa-1,4-glucano-proteína sintasa [formadora de UDP]. Estas seis proteínas se propusieron como marcadores posibles marcadores de resiliencia, lo que

las hace especialmente interesantes para su validación en experimentos posteriores, y deben ser confirmadas para un mayor número de poblaciones e individuos antes de ser consideradas como generales para la especie *Q. ilex*.

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